Two novel temperate bacteriophages co-existing in Aeromonas sp. ARM81 – characterization of their genomes, proteomes and DNA methyltransferases

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Aeromonas species are causative agents of a wide spectrum of diseases in animals and humans. Although these bacteria are commonly found in various environments, little is known about their phages. Thus far, only one temperate Aeromonas phage has been characterized. Whole-genome sequencing of an Aeromonas sp. strain ARM81 revealed the presence of two prophage clusters. One of them is integrated into the chromosome and the other was maintained as an extrachromosomal, linear plasmid-like prophage encoding a protelomerase. Both prophages were artificially and spontaneously inducible. We separately isolated both phages and compared their genomes with other known viruses. The novel phages show no similarity to the previously characterized Aeromonas phages and might represent new evolutionary lineages of viruses infecting Aeromonadaceae. Apart from the comparative genomic analyses of these phages, complemented with their structural and molecular characterization, a functional analysis of four DNA methyltransferases encoded by these viruses was conducted. One of the investigated N6-adenine-modifying enzymes shares sequence specificity with a Dam-like methyltransferase of its bacterial host, while another one is non-specific, as it catalyzes adenine methylation in various sequence contexts. The presented results shed new light on the diversity of Aeromonas temperate phages.

INTRODUCTION

Bacteriophages are among the most abundant biological entities on Earth (Weinbauer, 2004). They have an important impact on bacterial genomes and significantly contribute to the horizontal gene transfer (Canchaya et al., 2004). The majority of the sequenced bacterial genomes contain prophages that can constitute as much as 20% of the chromosomal gene content (Casjens, 2003). Prophages may dramatically change the phenotype of the host via lysogenic conversion which may provide new functions, such as increased virulence or improved fitness (Fortier & Sekulovic, 2013). Prophage DNA is usually physically integrated into the host chromosome. However, some phages, e.g. P1, LE1, f20, fBB-1 (Casjens, 2003), and vB_BceS-IEBH (Smeesters et al., 2011) are maintained in the prophage state as plasmids.

A unique group of plasmid-like prophages are those replicating as linear DNA with covalently closed ends (called telomeres). Apart from ‘a prototype’ – the enterobacteria phage N15, which was isolated in 1964 – only six other phages are known to lysogenize their hosts as linear telomere plasmids (Ravin, 2015). They are the siphoviruses ΦK02 of Klebsiella oxytoca (Casjens et al., 2004) and PY54 of Yersinia enterocolitica (Hertwig et al., 2003), and the myoviruses of marine bacteria: VP882, Vp58.5 and vB_VpaM_IEBH (Lan et al., 2009; Zabala et al., 2009; Alanis Villa et al., 2012), and ΦHAP-1 of Halomonas aquamarina (Mobberley et al., 2008). All of them encode a hallmark protein called protelomerase, along with plasmid partitioning proteins ParA and ParB, required for this type of lysogeny. Another myovirus, VHML of Vibrio harveyi (Oakey et al., 2002) that is closely related to one of the abovementioned phages, VP58.5 (Zabala et al., 2009)
also contains genes encoding homologues of the N15-like protelomerase (Ravin, 2015) and partitioning protein ParA (Oakley et al., 2002). VHML was initially described as a prophage integrated into the host chromosome (Oakley et al., 2002), but later it was suggested that it may be a linear plasmid-like prophage as well (Lima-Mendez et al., 2008). VHML was recently included into the family of N15-like linear phage-plasmids (Ravin, 2015).

_Aeromonas_ species are facultatively anaerobic, Gram-negative bacteria, ubiquitous in various environmental niches, including all types of water, food and soil. They are common pathogens of poikilothermic animals, and some of them are also linked with human diseases (Igbinosa et al., 2012). To date, genomes of 16 phages infecting _Aeromonas_ have been reported. Most of them are classified to the Podoviridae family (Kim et al., 2012). All the other known _Aeromonas_ phages, except for _ΦO18P_ (Beilstein & Dreiseikelmann, 2008), demonstrated a lytic lifestyle.

In this study, we have isolated and characterized two temperate phages of the _Aeromonas_ sp. ARM81; one chromosomally integrated, named _ΦARM81mr_ (alias _vBA_Asp81M_ARM81mr_ according to the new nomenclature system) and the other named _ΦARM81ld_ (_vBA_Asp81M_ARM81ld_) maintained extrachromosomally as a linear plasmid-like prophage with a protelomerase gene.

**RESULTS AND DISCUSSION**

Identification of prophage sequences in the draft genome of _Aeromonas_ sp. ARM81

The draft genome of _Aeromonas_ sp. ARM81 was determined. The total length of the genome was estimated as 4.6 Mb and the calculated G+C content was 61.3 %. We searched the genome for putative prophage sequences using the PHAST program. The analysis revealed the presence of two such regions, within contigs 1 and 51, respectively. In the case of the contig 51, the PHAST program suggested that the entire DNA segment is, in fact, a phage. Moreover, the genome assembly implied that this contig is extrachromosomally localized. To verify whether the recognized prophage regions are active phages, we treated the cells of _Aeromonas_ sp. ARM81 with mitomycin C, a classical inducer of lambdoid prophages. The exponentially growing culture of _Aeromonas_ sp. strain ARM81 was exposed to the mitomycin C, incubation was then continued and monitored by the OD<sub>600</sub> measurement. A dramatic decrease of optical density (below 0.1) was observed after about 2 h. The resulting lysate was purified by PEG precipitation and CsCl density gradient separation.

Interestingly, this approach caused the induction of both phages, _ΦARM81mr_ (representing an integrated, temperate prophage identified in contig 1) and _ΦARM81ld_ (representing a predicted extrachromosomal prophage identified in contig 51), respectively, each of which was located in the separate band on the CsCl gradient. Both phages were resequenced and characterized in this work.

It is noteworthy that during the extended cultivation time of the ARM81 strain growing in solid and liquid media, spontaneous phage induction occurred. The viral particles released in this way were mainly _ΦARM81mr_, which was confirmed by isolation of the phage DNA.

Several _Aeromonas_ spp. strains (see Methods) were tested as potential hosts for the _ΦARM81mr_ and _ΦARM81ld_ phages by a spot test. None of the tested bacterial strains supported detectable lytic growth of either of the phages. Therefore, we concluded that _ΦARM81mr_ and _ΦARM81ld_ are highly specific and have a narrow host range, possibly confined to its natural host strain ARM81.

**Morphology of the _ΦARM81mr_ and _ΦARM81ld_ phages**

TEM analysis showed that a virion of _ΦARM81mr_ had an icosahedral head about 60 nm in diameter and a tail about 70 nm long and 14 nm width, while _ΦARM81ld_ had an icosahedral capsid of 60–80 nm and a contractile tail of about 170 nm. These morphological features indicate that both viruses belong to the _Myoviridae_ family (Fig. 1).

**General features of the _ΦARM81mr_ and _ΦARM81ld_ genomes**

The nucleotide sequences of both prophages were deduced from the whole-genome shotgun library of _Aeromonas_ sp. ARM81 and confirmed by resequencing of the phage DNA isolated from the purified viral particles.

Sequence analysis showed that the genome of the phage _ΦARM81mr_ was 60 012 bp DNA with a G+C content of 60.9 %, which encoded 70 genes. Deduced amino acid (aa) sequences of all the proteins were compared with the

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**Fig. 1.** Transmission electron micrograph of the phages _ΦARM81mr_ (a) and _ΦARM81ld_ in uncontracted (upper part) and contracted (lower part) states (b). Samples were stained with 2 % uranyl acetate. The scale bar represents 100 nm.
known sequences using the BLAST program. Positions, sizes and putative functions of the proteins, and their closest known homologues, are listed in Table S1 (available in the online Supplementary Material). Based on the in silico analysis, we were able to assign putative biological functions to 34 genes (48%), while the remaining 36 genes encoded putative proteins with homology to the so-called hypothetical proteins (Table S1). Further analysis of the \( \Phi ARM81 \) genome revealed its modular structure, as we were able to distinguish putative genetic modules that encode functions crucial for the phage life cycle (Fig. 2). No evidence could be found for the presence of cohesive ends (cos) in the \( \Phi ARM81 \) genome, as heating of the viral DNA digested with restriction enzymes (REases) to 70°C, did not alter the banding pattern. Therefore, it can be assumed that the phage DNA was packaged by a headful mechanism (pac type) (Casjens & Gilcrease, 2009).

The location of the \( \Phi ARM81 \) prophage in the genome of Aeromonas sp. ARM81, determined by draft-genome sequencing of this strain, was confirmed by PCR amplification of the region across the junctions between the host and prophage genomes. Since the prophage is flanked by duplication of the 19-bp sequence (CCATGCCGGGGTAGTA-GAT), most likely, it constitutes the bacterial attachment site (attB). Integration of \( \Phi ARM81 \) into the genome disrupted a gene encoding a hypothetical protein of an unknown function, conserved in Aeromonas spp.

The second identified phage, \( \Phi ARM81ld \) was predicted to be an autonomous plasmid-like virus. The size of its genome is 47457 bp and its G+C content (58.6%) is significantly lower than the average for the host genome (61.3%).

The analysis of the \( \Phi ARM81ld \) DNA revealed alteration of the restriction pattern in the restriction enzyme analysis after the heat treatment of the samples, suggesting the presence of a cos site (data not shown). To identify the sequence of cos sites, the ligation mixture of phage ends (native or previously treated with the T4 polymerase) were used as templates in PCR reactions with primers ARM81ld_cosF and ARM81ld_cosR (Table S2). The amplified PCR products were cloned into the SmaI site of the vector pBluescript KS and sequenced. Comparison of the sequencing results indicated that the cos sites had 12-nt 5’ overhangs (GGGGTGTTAGACC).

The \( \Phi ARM81ld \) genome contained 61 putative genes. A total of 39 of the genes (66%) shared a significant similarity at the protein level with the other sequences in GenBank. Also, 15 genes are transcribed leftwards and 46 rightwards (Fig. 2). Putative functional assignments and significant similarities to the predicted genes are listed in Table S3. Interestingly, the closest homologues (best Blast hits) of as much as 39 (64%) \( \Phi ARM81ld \)-predicted proteins were Aeromonas popoffii-encoded hypothetical proteins. This finding may suggest that this particular species contains a prophage sequence similar to \( \Phi ARM81ld \) (Table S3).

Furthermore, roughly in two-thirds of the length of the \( \Phi ARM81ld \) genome (coordinates 33176–33259) there was a long (84-bp) palindromic sequence similar to the telomere resolution sites (telRL) of the known linear telomere phages. Only 100 base pairs downstream from the 84-bp palindrome, the gene \( ARM81ld_p43 \) was identified. It encoded a product showing similarity to the putative protelomerase of Vibrio phages VP58.5 (GenBank accession number CAX65021, 35% identity), vB_Vpa_MASTER (YP_007125175, 37% identity), VHML (NP_758894, 34% identity), VP882 (YP_001039865, 27% identity), and Halomonas phage PHAP-1 (YP_001686770, 26% identity). The presence of a typical module of linear plasmid-like phages indicated that \( \Phi ARM81ld \) most likely belongs to the same group of viruses.

The gene order in virion and prophage DNAs of N15 (and other telomere phages) is circularly permuted (Ravin, 2015). After circularization of virion DNA via its cohesive termini following infection, the protelomerase (TelN) cuts the telRL sequence, producing a linear molecule. Therefore telL and telR sequences that are adjacent to each other in virion DNA, are located at opposite ends in prophage DNA (Ravin, 2003). We took advantage of this fact to prove linear status of the \( \Phi ARM81ld \) prophage using combined restriction and Southern blot analysis. Fig. 3 shows that both molecular probes (PCR products of regions flanking telRL, coordinates: 32154–32627 and 33881–34439) hybridize to one restriction fragment of \( \Phi ARM81ld \) virion DNA, while in total DNA, the probes hybridize to respective, smaller restriction fragments of \( \Phi ARM81ld \) prophage DNA. The summarized length of both smaller restriction fragments of \( \Phi ARM81ld \) prophage DNA corresponds to the length of one restriction fragment of \( \Phi ARM81ld \) virion DNA (Fig. 3).

### Genetic modules of the \( \Phi ARM81mr \) phage

Within the genome of the \( \Phi ARM81mr \) phage, nine genetic modules were distinguished and their biological functions were predicted (Fig. 2). The integration and excision module of the \( \Phi ARM81mr \) phage is composed of two genes, encoding putative integrase (Int) and excisionase (Xis). The predicted Int protein (\( ARM81mr_p01 \)) belongs to the tyrosine recombinase family (Grainge & Jayaram, 1999). The closest homologues were found in the genomic islands AsaGE11b (accession number AIZ49611, 93% identity) and AsaGE11a (AIZ49548, 92.5%) of Aeromonas salmonicida HER1085 and 01-B526 (Emond-Rheault et al., 2015a), respectively. Interestingly, the homologues of \( ARM81mr_p01 \) are also encoded within the Shiga toxin (Stx)-converting phages of enterobacteria 933W, Min27, VT-2-Sakai, P13374, Stx2-I, and Stx2-II (Table S4). Downstream of the integrase gene \( ARM81mr_p01 \), a functional counterpart of the excisionase gene was identified (\( ARM81mr_p02 \)). Its protein product showed 88% identity with a putative Xis of the aforementioned genomic island AsaGE11b (accession number AIZ49612). The Xis protein works in cooperation with an
Replication machinery (Mallory origin the DnaB helicase, a key component of the cellular protein promotes phage replication by recruiting to the viral genome during the initiation of replication, whereas the P protein interacts with the origin of replication (\(\text{ori} \)) of the genome during the initiation of replication, whereas the P protein-promotes phage replication by recruiting to the viral origin the DnaB helicase, a key component of the cellular replication machinery (Mallory et al., 1990). Both putative replication proteins of \(\Phi\)ARM81mr are related to their equivalents in the enterobacteria phages 933W and Stx2-I (Table S4).

The lysis module of \(\Phi\)ARM81mr is composed of two genes. The protein encoded by \(\text{ARM81mr}_p42\) was classified as a member of the holin superfamily III. The second gene, \(\text{ARM81mr}_p43\), encoded a protein (putative endolysin) that contains the cysteine, histidine-dependent amidohydrolases/peptidases domain (CHAP domain, amino acids 39–113), which seems to be characteristic for peptidoglycan hydrolases (Ridgen et al., 2003).

The phage DNA packaging module of \(\Phi\)ARM81mr contains genes encoding both subunits of a terminase, which is typically a heterodimer composed of a smaller subunit with DNA-binding activity (\(\text{TerS}\)) and a larger subunit (\(\text{TerL}\)) with ATPase and endonuclease activity (Catalano, 2000). The predicted product of the \(\text{ARM81mr}_p47\) gene shows 39.9\% identity to the \(\text{TerS}\) protein of the Stx-converting enterobacteria phages and the Shigella phage POCJ13. The \(\text{ARM81mr}_p48\) gene encoded a protein homologous (~65\% identity) to \(\text{TerLs}\) from the abovementioned Stx-converting phages (Table S4). The phylogenetic analysis of the aa sequences of \(\text{TerLs}\) of \(\Phi\)ARM81mr and 11 related phages of enterobacteria (Min27, 933W, VT2phi_272, TL-2011c, VT2-5a, P13374, Stx1, Stx2-I, Stx2-II, 86, vB_EcoP_24BP13374) and Shigella (POCJ13, Stx1-HUN/2013) revealed that \(\text{ARM81mr}_p48\) formed an outgroup in relation to the other terminases, which proves their distant relationship (data not shown).

The packaging module is usually followed by a morphogenesis/structure module, but sequences encoding known phage structural proteins were not found within the \(\Phi\)ARM81mr genome, with the exception of the portal (\(\text{ARM81mr}_p49\)) and tail tip fiber (\(\text{ARM81mr}_p63\)) proteins. To obtain a more comprehensive view of the...
 ARM81mr structural region SDS–PAGE (Fig. 4a), mass spectrometry analysis of the capsid proteins was carried out. Proteomic characterization of the virion particles allowed for identification of 11 ARM81mr-encoded products (ARM81mr_p49, _p52, _p53, _p56, _p57, _p62, _p63, _p65, _p66, _p68 and _p69, respectively). Among them were nine polypeptides, initially annotated as hypothetical/uncharacterized proteins, which can now be described as virion-associated proteins (Table S1). Homologues of almost all of these structural proteins (like the aforementioned terminases) are encoded by Stx-converting phages (Table S4).

The ARM81mr phage also carries two additional genetic modules, which are not directly linked with the phage ‘life cycle’. The protein encoded by the ARM81mr_p59 gene exhibits an aa sequence homology with RNA-directed DNA polymerases (reverse transcriptases), which are components of self-splicing, bacterial mobile genetic elements called ‘retroelements’ (Toro et al., 2007).

Genetic modules of the ARM81ld phage

Within the genome of the second Aeromonas sp. ARM1 phage, the plasmid-like, linear virus ARM81ld, eight genetic modules were distinguished. The biological functions of the predicted modules are as follows: packaging, morphogenesis, partitioning, replication, lysogeny control, lysis, DNA methylation and transposition (Fig. 2).

Within the packaging module, two genes (ARM81ld_p01 and _p02), encoding putative TerS and TerL, were identified. ARM81ld_p02 was most similar (40 % identity) to its functional TerL counterparts of the enterobacteria.
phages, e.g. λ (37.2 % identity). The other protein, ARM81ld_p01, seemed unique as it showed no significant similarity to any small-subunit of the known terminases, even though it contains a Nu1 domain, characteristic for these enzymes.

Mass spectrometry was used to confirm the identification of the structural proteins encoded in the ΦARM81ld morphogenesis module (Fig. 4b). Seven structural proteins were identified this way: portal (ARM81ld_p04), head decoration (ARM81ld_p06), major capsid (ARM81ld_p07), baseplate assemblies W and J (ARM81ld_p13 and p14), tail tube FI (ARM81ld_p19) and tail sheath FII protein (ARM81ld_p20). The close homologue of ARM81ld_p07 is the major capsid protein (MCP) of N15 phage (NP_046903, 43.3 % identity). The latter protein did not have any relatives among MCPs of telomere phages until now. Other predicted ΦARM81ld head morphogenesis gene products also share similarity with their functional counterparts of the phage N15 (Table S5). In turn, ΦARM81ld tail structure and morphogenesis gene products are homologous to their functional counterparts of ΦHAP-1 and VP882 phages which is not surprising, because together with ΦARM81ld, they belong to the Myoviridae family (Table S5).

Another two modules identified within the ΦARM81ld genome are responsible for the maintenance of the virus within the host cells. ARM81ld_p41 and p42 gene products have been identified as putative components of the plasmid partitioning system (ParABS). ParABSs are encoded by many low copy-number plasmids and bacterial chromosomes. They interact with one or more cis-acting sites, termed centromere-like sequences, to ensure an even distribution of the DNA molecules to daughter cells during cell division (Gerdes et al., 2000). Putative ParB (ARM81ld_p41) and ParA (ARM81ld_p42) proteins were related to their functional counterparts of the linear phages ΦK02 (39.5 % and 51.8 % identity, respectively), PY54 (37.2 % and 50.1 % identity) and N15 (37 % and 51.5 % identity), whereas a putative replication protein (ARM81ld_p45) exhibited similarity with its equivalents found in other plasmid-like prophages, namely the virophages vB_VpaM MAR (42 % identity), and VP58.5 (40 % identity) and also VHLM (41 % identity) (Table S5).

The linear plasmid-like phages, similarly to other temperate viruses, possess genes responsible for switching between the lytic and the lysogenic cycle (Hammerl et al., 2015). Such a control region was found in the ΦARM81ld phage. The topological homologues of cl and cro genes are ARM81ld_p46 and ARM81ld_p47, respectively. The product of ARM81ld_p46 contains a conserved helix-turn-helix DNA-binding domain and a peptidase S24-like domain (pfam0071). It also shares similarity with prophage repressor proteins of linear phages PY54 (33.5 % identity), ΦK02 (25.7 % identity), N15 (27.5 % identity), while the predicted Cro-like ARM81ld_p47 protein showed no significant similarity to any sequence present in the Uniprot viral database.

The lysis module of the phage ΦARM81ld contained a putative holin (ARM81ld_p57), lysis (ARM81ld_p58), and two other genes, encoding putative Rz/Rz1-like lysis accessory proteins (ARM81ld_p59-p60). It is worth noting that ARM81ld_p58 also shows 75 % aa identity with a putative endolysin ARM81mr_p43 of the ΦARM81mr phage, which may suggest that both temperate phages of Aeromonas sp. ARM81 use similar lytic enzymes in order to disintegrate the cell wall of the host.

The last gene identified within the ΦARM81ld genome ARM81ld_p27 encodes a protein homologous to the transposases of insertion sequences belonging to the IS200/IS605 family (Siguier et al., 2006). Interestingly, BLAST searches (ISfinder) revealed that this protein shows the highest level of aa sequence identity (36 %) with the transposase of an archael insertion sequence, ISMor19, of Methanosarcina mazei Go1 (Deppenmeier et al., 2002).

Comparative genomics of ΦARM81mr and ΦARM81ld

In general, the ΦARM81mr genome shares little nucleotide-level identity with any bacteriophage genome currently available in the NCBI database (as of 25-08-2015). However, a small section of the genome (351 bp, coordinates 28892–29443) shows 74 % identity with the Shigella phage POCJ13 (Gray et al., 2014). This region contains a fragment of a putative portal gene of ΦARM81mr (ΦARM81mr_p49).

In the course of this study, several putative prophages related to ΦARM81mr were detected in the genomes of Aeromonas hydrophila strains AH10 (accession number NZ_CP011100), AL06-06 (Tekedar et al., 2015), J-1 and NJ-35 (Pang et al., 2015). Their shared homologues were encoded in a collinear cluster of genes and included integrase, excisionase (in NJ-35 only), recombination proteins, replication proteins P and O, holin, endolysin, both terminases, portal and other structural proteins (Fig. 5a, Table S4).

A significant similarity between ΦARM81mr and four genomic islands of A. salmonicida strain HER1085 (AsaGE11b), 01-B526 (AsaGE11a), JF3224 (AsaGE12b), 09-0167 (AsaGE12a) (Emond-Rheault et al., 2015a, 2015b) was also observed (Fig. 5b, Table S4). It covered almost exclusively the left arm of the ΦARM81mr genome without the transcription regulation module of the lytic–lysogenic switch (cl-cro-antirepressor genes). The right arm of ΦARM81mr seemed to be unrelated to the abovementioned genomic islands, except for a relatively long gene ARM81mr_p69, encoding a hypothetical structural virion protein, whose presence in capsids was confirmed by proteomic analysis (Fig. 4a). This gene was also conserved in putative prophages of A. hydrophila (Fig. 5a, Table S4) and homologous proteins were encoded by Stx-converting phages (Table S4).

Interestingly, it was also found that other ΦARM81mr-encoded proteins shared homology with respective proteins of Stx-converting enterobacteria and Shigella bacteriophages.
Fig. 5. Comparative genomic analysis of the ΦARM81mr phage and prophages identified within Aeromonas hydrophila genomes (a) and genomic islands present within Aeromonas salmonicida genomes (b). The grey-shaded areas connect DNA regions of different prophages/genomic islands with at least 70% nucleotide sequence identity. The genomic coordinates for
the particular prophages of A. hydrophila are as follows: prophage 1: 2 350 001–2 416 400 and prophage 2: 2 416 401–2 465 700 of AH10 (accession number NZ_CP011100); prophage 1: 1 917 893–1 981 545 and prophage 2: 2 449 400–2 511 100 of NJ-35 (NZ_CP006870); prophage of AL06-06 (NZ_CP010947): 1 647 382–1 700 000; prophage of J-1 (NZ_CP006883): 2 379 808–2 441 507. The nucleotide sequence of the prophage 1 of NJ-35 predicted by us, overlaps the prophage regions determined by Pang et al., 2015. [NJ-35 prophage-2 (1 917 892–1 944 140) and NJ-35 prophage-3 (1 935 562–1 981 545)]. The nucleotide sequence of the prophage 2 of NJ-35 predicted by us overlaps the prophage region determined by Pang et al., 2015. [NJ-35 prophage-5 (2 481 289–2 510 319)].

(see above). Among the shared proteins were: integrase, DNA methyltransferase, DNA replication and recombination (protein O and P, NinB), transcription regulation (CI, Cro and antirepressor), packaging enzymes (terminases and portal protein), bacteriocin and structural proteins (Table S4).

Comparison of the FARM81ld genome with phage genome sequences available in the NCBI database showed no discernible DNA sequence similarity to any of them. However, the gene content and structural organization of several functional FARM81ld modules were similar to all of the previously described N15-related phages. BLASTP analyses showed that the FARM81ld proteome shared 15 homologues (24.6 % of FARM81ld-encoded proteins) with VP882, 13 (21.3 %) with VP58.5, vB_VpaM_MAR and FARM-1, 12 (19.7 %) with VHML, 11 (18 %) with N15, 9 (14.8 %) with PY54 and 5 (8.2 %) with FARM-2. The analysis of the homologies between FARM81ld and other aforementioned phages confirmed that it is a mosaic virus, sharing homologies with both enterobacterial (e.g. partitioning proteins, CI repressor) and marine (e.g. replication proteins) N15-related phages (Fig. 6).

All of these phages also encoded homologous protelomerases, which were used for phylogenetic analysis. It revealed that the FARM81ld-encoded protein forms an outgroup in a cluster grouping vB_VpaM_MAR, VHML, VP58.5 protelomerases, and is relatively distant to other enzymes encoded by the analysed linear phages (Fig. 6), which supports conclusion of its uniqueness.

Functional analysis of the DNA methylation enzymes of FARM81mr and FARM81ld

Whole-genome sequencing and in silico analysis of the FARM81mr and FARM81ld genomes revealed several putative (orphan) methyltransferase (MTase)-encoding

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Fig. 6. Phylogenetic analysis of the TelN-like proteins and homologies between FARM81ld proteins and the proteins of other N15-related phages. The unrooted tree was created using the maximum likelihood algorithm. Statistical support for the internal nodes was determined by 1000 bootstrap replicates. Values above 50% are shown. The accession numbers of phages encoding particular protelomerases are given in parentheses. The homologies between FARM81ld proteins and the proteins of other N15-like phages were presented in a binary (0 : 1) mode, where black and white rectangles show presence or lack of homology, respectively. Searches were performed with a cut off e-value $1 \times 10^{-10}$ and at least 50% query coverage. The particular genetic modules within FARM81ld were presented (PAC, packaging; S&M, structural and morphogenesis; TE, transposable element; $m^5$C MET, $m^6$C cytosine methylation; PAR, partitioning; REP, replication; LC, lysogeny control; $m^5$A MET, adenine DNA methylation; LS, lysis).

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genes. The predicted protein products of the ARM81mr_p29 and ARM81ld_p31 MTases, including M. Gel16401IV of Geopseudorhabdobacter electrodiphilus DSM 16 401, whose target motif (CCAG, the methylated base is underlined) was identified by SMRT sequencing technology [according to REBASE, (Roberts et al., 2015)]. ARM81mr_p29 and ARM81ld_p31 were in 58 % identical and shared 56 % and 46 % identity with M.Gel16401IV, respectively. Plasmid DNA of pET-ARM81mr_p29 and pET-ARM81ld_p31 (carrying the ARM81mr_p29 and ARM81ld_p31 genes, respectively) was isolated from the E. coli ER2566 strain grown in the presence of IPTG, which induces recombinant protein expression. To define sequence specificity of both MTases, a panel of cytosine methylation-sensitive endonucleases in REase cleavage interference assays was used. REases which are m^C-insensitive were used as controls to confirm that the potential interference is m^C specific. The DNA of pET-ARM81mr_p29 and pET-ARM81ld_p31 isolated from induced cells was partially resistant to EcoRII (CCWGG), Bme1390I (CCNGG) and HpaII (CCGG), but all the other REases cleaved the same DNA to completion. No interference was seen for the same plasmid isolated from the non-induced cells (Fig. S1 and S2, available in the online Supplementary Material).

Surprisingly, the DNA of both phages isolated from virions seemed to be cleaved completely by EcoRII, Bme1390I and HpaII REases (data not shown) suggesting that these enzymes are not produced during the replicative cycle of the virus. It is still possible that these enzymes are present only temporarily and might be involved in the regulation of the timing of a cellular or phage process, which takes place before phage DNA replication, or that they are produced during replication, but the expression level is not sufficient to be detected in the REase interference assay.

The analysis of the genome sequence of ßAR81mr also revealed the presence of a putative type II N6-adenine (m^A) DNA MTase (ARM81mr_p11), which was highly similar to many Dam-like proteins, e.g. M. Aca8LMORFDP (accession number WP_045525482, 96 % identity) of Aeromonas caviae 8LM (Moriel et al., 2015). To determine whether GATC sequences are substrates for ARM81mr_p11, the protein was overexpressed in E. coli ER2566 and after purification, used for in vitro methylation of phage λ DNA (dam^-, dcm^+). The status of this methylation was subsequently tested by incubating the treated DNA with an excess of the following REases: DpnI (requires adenine methylation of GATC sites for cleavage), MboI (inhibited by m^A methylation), Bsh1236I and MspI (controls, the enzymes are not sensitive to m^A methylation). A DNA treated with ARM81mr_p11 was sensitive to all the restriction enzymes except for MboI, which indicates adenine modification in the GATC sequence (data not shown).

To assess whether there are other sites (in addition to GATC) in the λ DNA which could be modified by ARM81mr_p11, the incorporation of radiolabeled methyl group from S-adenosyl methionine into the λ DNA, pre-digested with MboI-restriction endonuclease was measured. No incorporation was detected in this assay, while the intact λ DNA or the same DNA cleaved with HindIII was apparently modified by the ARM81mr_p11 enzyme (Table 1). Based on these results, we conclude that ARM81mr_p11 has a specificity towards GATC sequences and, unlike some other DNA MTases, does not show visible substrate promiscuity (Clark et al., 2012; Aranda et al., 2012).

It should be noted that the DNA of the ßAR81mr phage was subjected to various REases sensitive to m^A modification (REBASE, (Roberts et al., 2015)), including DraI (TTTAAA), VspI (ATTAAT), Tasi (AATT), SpI (AATATT), Hin1III (CATG), HinfI (GANTC) and TruI (TTAA), but resistance to cleavage was only observed in the case of MboI (data not shown). However, it is unclear whether this is the result of the activity of ARM81mr_p11 or a cellular Dam-like enzyme. Aeromonas sp. ARM81 contains a homologue of the A. hydrophila YLI7 MTase (M.AhyYL17Dam, 95 % identity), which modifies the

<table>
<thead>
<tr>
<th>Sample</th>
<th>Methylation level (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control: no enzyme</td>
<td>180</td>
</tr>
<tr>
<td>Negative control: enzyme heated to 80 °C for 15 min before the reaction</td>
<td>180</td>
</tr>
<tr>
<td>λ DNA cut with MboI methylated by ARM81mr_p11</td>
<td>300</td>
</tr>
<tr>
<td>λ DNA cut with HindIII methylated by ARM81mr_p11</td>
<td>23 500</td>
</tr>
<tr>
<td>uncut λ DNA methylated by ARM81mr_p11</td>
<td>24 000</td>
</tr>
<tr>
<td>λ DNA cut with MboI methylated by Hia5*</td>
<td>140 000</td>
</tr>
<tr>
<td>λ DNA cut with HindIII methylated by Hia5</td>
<td>155 000</td>
</tr>
<tr>
<td>Uncut λ DNA methylated by Hia5</td>
<td>169 000</td>
</tr>
</tbody>
</table>

*The sequence specificity of m^A MTase Hia5 is BA (where B=C, G or T), so it possessed the ability to methylate almost all adenine residues in DNA (Drozdz et al., 2012). We used this enzyme as a control to show that in the λ DNA cut with MboI, there are other potential sites to be methylated by the m^A MTase.
adenine residue in the sequence GATC (Chan et al., 2014). The chromosomal DNA of Aeromonas sp. ARM81 was not cut by MboI (data not shown), which indicates the presence of GATC-specific modification enzyme in the host cells prior to phage induction.

Dam MTases are widely distributed among Gammaproteobacteria (Reisenauer et al., 1999) and have been reported to regulate chromosome replication, transcription and mismatch repair (Marinus & Casadesus, 2009). Moreover, GATC methylation plays a role in the virulence of diverse pathogens, e.g. Salmonella enterica serovar Typhimurium, Vibrio cholerae, Yersinia pestis, and Yersinia pseudotuberculosis (Heithoff et al., 1999; Julio et al., 2001; Robinson et al., 2005). It has also been shown that the dam gene is essential for the viability of A. hydrophila SSU and that M. AhySSU-Dam is involved in A. hydrophila pathogenesis (Erova et al., 2006a, b, 2012). The putative Dam-like protein of Aeromonas sp. ARM81 showed 94 % identity with the abovementioned protein.

Genes encoding Dam-like MTases are also found in the genomes of other bacteriophages, e.g. P1 (Coulby & Sternberg, 1988), VT-2 (Radlinska & Bujnicki, 2001) and T-phages of E. coli (Scherzer et al., 1987), HP1 and HP2 of Haemophilus influenzae (Bujnicki et al., 2001; Piekarowicz & Bujnicki, 1999), but their function remains unclear. It must be emphasized that in all these cases, phage-encoded enzymes co-exist with Dam MTases of the host. ΦARM81m1 is another example of gammaproteobacterial virus mimicking the host strategies in DNA methylation. This phenomenon has also been discovered recently for Alphaproteobacteria phages (Dziewit et al., 2014a, b).

A putative type II m^A MTase was also identified in the ΦARM81ld genome. The protein product of the ARM81ld_p56 gene was similar to M. EcoGII (52 % identity) and M. EcoGII (52 % identity) of a pathogenic E. coli strain O104: H4 C227-11 (Rasko et al., 2011). Both these MTases non-specifically methylate adenine residues in most sequence contexts and are active when exogenously expressed, but they have low, or no activity in the host cells (Fang et al., 2012). The first indication that substrate specificity of ARM81ld_p56 is also relaxed was obtained from an endonuclease protection assay. Plasmid DNA of pET-ARM81ld_p56 isolated from an IPTG-induced E. coli ER2566 was partially resistant to cleavage with REases sensitive to m^A in their recognition sequences, e.g. HinII, (GANTC), HinHI (CATG), TruII (TTAA) and TaqI (AATT) (Fig. S3). In the second test of sequence preferences of ARM81ld_p56, a set of oligonucleotide duplexes, which were previously applied to confirm extraordinary sequence promiscuity of the DNA:m6A MTase Hia5 (Drozdz et al., 2012), was used as a substrate. Each of the duplexes contained repetitions of a dinucleotide, in which adenine was accompanied by another nucleotide (i.e. CA, GA, TA and AA). An example of methylation reaction is shown in Fig. S4. Increase of radiolabel incorporation was linear for ~15 min. Substrates with repetitions of the dinucleotides TA and GA (TA30, GA10 respectively, Table S6) were methylated with comparable rates by ARM81ld_p56, and CA was methylated twice, and AA five times slower than TA and GA (Fig. 7). These results suggest that all adenine residues in a double-stranded DNA constitute potential substrates for ARM81ld_p56, and that this enzyme has no sequence specificity.

ARM81ld_p56 shares sequence similarity with putative m^A MTases encoded by, among others, an Aeromonas temperate phage ΦO18P (Beilstein & Dreiseikelmann, 2008) (62.7 % identity), and six out of eight known N15-related phages, i.e. ФKO2, PY54, vB_VpaM_MAR, VHML, N15 and VP58.5 (Fig. S5, Table S5). It is possible that these enzymes also lack sequence specificity.

Expression of some prophage genes, also DNA-modification ones, may be harmful to the host and therefore, must be strictly controlled. An example of a very tight control of production of a DNA-modification enzyme is regulation of an operon containing overlapping com and mom genes, located at the rightmost end of an Enterobacteria phage Mu genome (Hattman, 1999). The mom gene encodes a protein responsible for the unusual da’x (α-N-(9-β-d-2’-deoxyribofuranosylpurin-6-yl)-glycinamide DNA modification, and whose untimely expression is cytotoxic. Com is a zinc finger site-specific mRNA-binding protein required for translation of the mom gene (Hattman et al., 1991; Wulczyn et al., 1989). It was shown that other Mu-like phages encoding Mom homologues (or m^A MTases occupying the same loci in the phase genomes) also contained Com-like regulatory proteins at corresponding positions within their genomes (Drozdz et al., 2012). Sequence analysis revealed the presence of an ARM81ld_p56-overlapping gene, encoding a Com-like protein (ARM81ld_p55), which shared sequence similarity with the Mu-encoded Com protein (accession number NP_050656, 43 % identity). Moreover, the putative Com
protein of ϕARM81ld contains four cysteines (aa residues 8, 11, 26 and 29) in positions analogous to Cys 6, 9, 26 and 29 of phage Mu Com (Fig. S6), which are required for the formation of a zinc finger (Witkowski et al., 1995). This suggests that ARM81ld_p53 plays the same role as Mu Com and controls the expression of a DNA-modifying enzyme, i.e. ARM81ld_p56.

‘Momification’ protects the Mu phage DNA from a variety of host-controlled restriction-modification systems and, as mentioned above, is harmful to the host, therefore it is late-activated in the Mu growth cycle, when the host cell is already destined for death (Hattman, 1999). Nevertheless, it does not seem that ϕARM81ld uses ARM81ld_p56 as its antirestriction strategy, as the ϕARM81ld genomic DNA turned out to be susceptible to cleavage by REases, which are sensitive to m^A modification (data not shown). However, it is still possible that ARM81ld_p56 is expressed at a very low level. On the other hand, it cannot be ruled out that ARM81ld_p56 activity represents a unique tactic to ensure the phage exclusion using methylation to interfere with DNA replication of superinfecting phages, preventing them from successfully initiating their infections and so phage progeny production. Suppressing of phage replication simultaneously blocks bacterial chromosome replication which consequently leads to bacterial death. The ‘suicide’ response to an infection by lytic phages is known in several bacteria species and represents an effective defense strategy to limit phage spread (Refardt et al., 2013; Snyder, 1995). Many phage-resistance strategies depend on the use of horizontally acquired ‘selfish’ elements (plasmids and prophages) that can provide efficient barriers to phage infection (Seed, 2015). Interestingly, orphan MTases were found to be much more abundant in temperate compared with virulent phages, which suggests that such MTases have a rather adaptive role in lysogeny, than protection of their DNA against the restriction endonuclease cleavage (Oliveira et al., 2014).

Although this novel non-specific MTase turned out to be active in the heterologous host, the molecular mechanism and biological importance of ARM81ld_p56 in the ARM81 strain remains to be determined.

CONCLUSIONS

While our knowledge on lytic phages of Aeromonas is quite vast, there is a gap in research in the area of temperate phages infecting this genus. Therefore, our study of two novel temperate phages of lysogenic strain ARM81 to some extent provides the missing information. These novel phages show no similarity to the previously characterized Aeromonas phages and might represent new evolutionary lineages of viruses infecting Aeromonadaceae. Interestingly, we were able to find blocks of homologous sequences between ϕARM81mr and the genomes of Aeromonas spp., which suggest the presence of related prophage sequences in other strains.

It is worth emphasizing that this work, to our knowledge, is the first report on the simultaneous induction of co-existing temperate bacteriophages in Aeromonas. Genome analyses of many genera, such as Bacillus, Enterococcus, Listeria, Mannheimia, Paracoccus, Staphylococcus and Streptococcus revealed that polylysogeny is a common phenomenon (Dziewit et al., 2014a; Goerke et al., 2009; Niu et al., 2015). Recently, we have also identified a polylysogenic Sinorhizobium sp. LM21, carrying two prophages (Dziewit et al., 2014b, 2015). As it was previously reported, usually only one phage can be recovered after induction of polylysogenic strains, or the productivity of at least one phage declines, which is probably a consequence of the competition between the co-infecting phages (Refardt, 2011; Niu et al., 2015). Unfortunately, the competition between prophages co-existing in one bacterial cell is an unexplored area of phage ecological research and needs further studies. The discovered system of two active temperate phages in a single host cell could be used as a model for studying such potential competitive interactions in the future. Moreover, the fact that the newly characterized phages use different variants of lysogeny, chromosomal integration versus extra-chromosomal plasmid-like replicon, might help to compare the potential success of either side of the lytic cycle.

One of DNA MTases characterized in the course of this study, ARM81ld_p56, was found to have no sequence specificity. As far as we know, this is the first work that shows the catalytic activity of m^A DNA MTase encoded by the N15-related phage. It should be stressed that six of eight phages belonging to this group, and ϕO18P posses gene-encoding proteins homologous to ARM81ld_p56. To our knowledge, it is also the first description of an active bacteriophage-carrying MTase which methylates adenine residues in many various contexts. So far, enzymes with such an extremely relaxed substrate specificity have been only found in defective prophages and phage-related elements (Drozdz et al., 2012; Fang et al., 2012). Like in the case of other non-specific adenine MTases, ARM81ld_p56 activity was not detected in the lysogenic strain. We hypothesize that this enzyme may be involved in a new type of phage defensive strategy using massive adenine-residue methylation to suppress replication of foreign phages and to block phage progeny production.

METHODS

Bacterial strains, plasmids, media, and growth conditions. The strains used in this study were Aeromonas sp. ARM81 [collection of Department of Bacterial Genetics; an environmental strain from a wastewater treatment plant in Warsaw (Poland)], Aeromonas caviae DSM-30025, DSM-30188 and DSM-7321 (purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures) and three environmental Aeromonas spp. from our collection, Escherichia coli TOP10 (Invitrogen) and ER2566 (New England BioLabs). The E. coli and Aeromonas strains were cultured under standard conditions in LB medium at 37 °C and 30 °C, respectively. When required, growth media were supplemented with kanamycin (50 µg ml ^−1), ampicillin (100 µg ml ^−1), and glucose (1%). The following plasmids were used in this work: pBluescript KS (Stratagene) and pET30a (Invitrogen).

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Standard molecular biology procedures. Standard DNA manipulations were carried out according to the protocols described by (Sambrouk & Russell, 2001). Total DNA was isolated from Aeromonas sp. ARM81 using a genomic DNA purification kit (Life Technologies). PCR reactions were performed with Phusion High-fidelity DNA polymerase (Life Technologies). Subsequently, the PCR products were digested with restriction enzymes and cloned into appropriate vectors. Restriction digest assay was performed in a 20-µl reaction volume under conditions recommended by the manufacturer using 0.3 µg of the DNA resulting suspension of the phages was mixed with CsCl (final concentration of 0.7 g ml⁻¹) and centrifuged at 150 000 g for 24 h at 4 °C. The visible viral bands were separately collected, diluted 1:10 in SM buffer (100 mM NaCl, 10 mM MgSO₄, 50 mM Tris-HCl, pH 7.5), and centrifuged for 2 h at 110 000 g at 4 °C. Pelleted bacteriophage particles were resuspended in SM buffer. Phage DNA was isolated by phenol–chloroform extraction and isopropanol precipitation (Sambrouk & Russell, 2001) and analysed by 0.7 % agarose gel electrophoresis.

Induction, purification of phage particles, and phage DNA preparation. Phages of Aeromonas sp. ARM81 were induced using mitomycin C. The bacterial culture was grown to an optical density of 0.4 at 600 nm (OD₆₀₀). The culture was then treated with mitomycin C (500 µg ml⁻¹), and its growth (with shaking) was continued until nearly complete lysis of the culture after 2 h. Phage particles were purified from the lysate by PEG/NaCl precipitation (Sambrouk & Russell, 2001). The resulting suspension of the phages was mixed with CaCl₂ (final concentration of 0.7 g ml⁻¹) and centrifuged at 150 000 g for 24 h at 4 °C. The visible viral bands were separately collected, diluted 1:10 in SM buffer (100 mM NaCl, 10 mM MgSO₄, 50 mM Tris-HCl, pH 7.5), and centrifuged for 2 h at 110 000 g at 4 °C. Pelleted bacteriophage particles were resuspended in SM buffer. Phage DNA was isolated by phenol–chloroform extraction and isopropanol precipitation (Sambrouk & Russell, 2001) and analysed by 0.7 % agarose gel electrophoresis.

Electron microscopy. Phage particles were negatively stained with 2 % uranyl acetate and electron micrographs were captured with a LEO 912AB transmission electron microscope (Zeiss) at 80 kV with a magnification of ×100 000.

DNA–DNA hybridization. The ΦARM81ld phage DNA fragments used as molecular probes were amplified by PCR using specific oligonucleotide primer pairs listed in Table S2 (ARM81ld_TelR1p and ARM81ld_TelF1p, ARM81ld_TelR2l and ARM81ld_TelF2l, respectively), gel-purified and labeled with digoxigenin (Roche). Hybridization and visualization of bound digoxigenin-labeled probes were carried out as recommended by the supplier (Roche). Total DNA isolated from Aeromonas sp. ARM81 and DNA isolated from CaCl₂-purified ΦARM81ld virions were digested with MplI103I, NdeI and Small REAs. Digest mixture were electrophoresed on 0.8 % agarose gels and stained with ethidium bromide. The same gel was used for Southern blot hybridization with abovementioned digoxigenin-labeled probes.

Phage structural protein analysis. Phage structural proteins were analysed by SDS-PAGE as previously described (Dziwen et al., 2014b). After electrophoresis, the protein bands were visualized by staining the gel with Coomassie blue R-250 dye and identified by liquid chromatography–tandem mass spectrometry (LC–MS/MS) in the Mass Spectrometry Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (IBB PAS, Warsaw, Poland).

Cloning, overexpression, purification, and testing the activity of putative DNA MTases. Four putative MTase genes were amplified from phage DNA using Phusion-HF DNA polymerase and gene-specific oligonucleotide primers (Table S2). PCR products were digested with NdeI (or VspI) and Xhol (or NotI), depending on the restriction sites added to the forward and reverse primers, and then ligated into a NdeI–Xhol-(or NdeI-Notl)-digested pET30a vector. The resulting plasmids encoded recombinant proteins with C-terminal His₆–tag. All the His₆–tagged recombinant enzymes were expressed in the E. coli strain ER2566. The proteins were purified by Ni-NTA affinity chromatography on a His-Select Nickel Affinity Gel (Sigma) as previously described (Drozdz et al., 2012). Restriction enzyme digestion-protection assay and radioactive DNA methyltransferase assay were performed as previously described (Drozdz et al., 2012). The pretreatment mixture contained 1 µM oligo duplex (Table S6), 4.6 µM [3H]AdoMet, and 500 nM recombinant protein ARM81ld_p56 in buffer M (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM 2-mercaptoethanol, 100 µg ml⁻¹ bovine serum albumin). Reactions were incubated at 20 °C for 10 min. Each methylation reaction was carried out at least in triplicate. Initial velocities of reactions were calculated from the slope of reaction progress curves.

DNA sequencing. Genomic DNA of the strain ARM81 was isolated using CTAB/Lysozyme method (Nettmann et al., 2008) and Illumina TruSeq library was constructed following the manufacturer’s instructions. The bacterial and phage genomes were sequenced on Illumina MiSeq instrument in paired-end mode using v3 chemistry kit. The obtained sequence reads were filtered for quality and assembled using Newbler v3.0 software (Roche).

Bioinformatics. The obtained phage nucleotide sequences were analysed using Clone Manager (Sci-Ed8) and Artemis software (Carver et al., 2008). The phage sequences within the draft genome were identified using PHAGE Search Tool (PHAST) (Zhou et al., 2011). Identified insertion sequence was analysed using the ISfinder database (Sigier et al., 2006). Similarity searches were performed using the blast program (Altschul et al., 1997) provided by the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the Simple Modular Architecture Research Tool (SMART) (Letunic et al., 2012), the UniProt (Apweiler et al., 2004) and Pfam databases (Finn et al., 2014). Putative tRNA genes were searched using the RNAscan-SE (Schattner et al., 2005) and aragorn programs (Laskett & Canback, 2004). Helix-turn-helix motifs were predicted using the helix-turn-helix motif prediction program (Dodd & Egan, 1990). Phylogenetic analyses were performed using MEGA (Tamura et al., 2011) with the maximum likelihood algorithm (1000 bootstrap replicates). The phylogenetic tree was rendered with TreeView version 1.6.6 (Page, 1996). For the visualization of the comparative genomics results the Easyfig program was used (Sullivan et al., 2011).

Nucleotide sequence accession numbers. The complete nucleotide sequences of the phages ΦARM81mr and ΦARM81ld have been deposited in the NCBI GenBank database under accession numbers KT898134 and KT898133, respectively.

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