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1	Novel Siphoviridae phage PMBT4 belonging to the group b Lactobacillus delbrueckii subsp.
2	bulgaricus phages
3	
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21	Highlights:
22	• A novel Cequinquevirus PMBT4 was identified from African fermented milk product by
23	electron microscopy and high-throughput sequencing (HTS).
24	• Complete genome sequence of the virus was obtained by HTS.
25	• The Cequinquevirus PMBT4 shares the highest NT identity with Lb. delbrueckii group b phage
26	c5.

- No KIS gene element was found in the genome of phage PMBT4 despite the presence of
   observed collar structures on some phages previously suggested to be encoded by these
   genes.
- 30

31 Abstract. A novel Lactobacillus delbrueckii bacteriophage PMBT4 was isolated from the Nigerian 32 fermented milk product nono. The phage possesses a long and thin, non-contractile tail and an 33 isometric head, indicating that it belongs to the Siphoviridae family. A neck passage structure (`collar`), previously hypothesized to be encoded by two genes located in the Lactobacillus 34 35 delbrueckii phage LL-K insertion sequence (KIS) element, as well as in two additional Lb. delbrueckii 36 phages Ld17 and Ld25A, could also be observed on an estimated 1-5% of phage particles by 37 transmission electron microscopy. However, neither mapping of high throughput sequencing data to 38 KIS element genes from Lb. delbrueckii phages LL-K, Ld17 and Ld25A nor PCR amplification of the KIS 39 element genes could corroborate the presence of these genes in the PMBT4 genome. The PMBT4 40 genome consists of 31,399 bp with a mol% GC content of 41.6 and exhibits high (95-96%) sequence 41 homologies to Lb. delbrueckii phages c5, Ld3, Ld25A and Ld17, which assigned PMBT4 as a new 42 member of this genus, i.e. the *Cequinquevirus* genus. 43

44 Keywords: Lactobacillus delbrueckii phage, genome, group b, KIS element

45

## 46 **1. Introduction**

*Nono* is a fermented cow milk product produced in the northern parts of Nigeria. It is predominantly prepared and sold on local markets by the Hausa/Fulani cattle herdsmen (Ogbonna, 2011). *Nono* is a spontaneously (lactic acid) fermented beverage (Okagbue and Bankole, 1992). So far, there have not been many studies performed on the diversity of LAB associated with *nono* fermentation. Banwo et al. (2012) showed that quite diverse lactic acid bacteria (LAB) are associated with *nono* production.

52 These LAB were identified as Lactiplantibacillus plantarum, Enterococcus and Pediococcus spp., while 53 Okagbue and Bankole (1992) identified Lactococcus lactis subsp. lactis biovar diacetylactis, 54 Levilactobacillus brevis and the yeast Saccharomyces cerevisiae to be associated with the 55 fermentation. Knowledge of the bacteria involved is, however, a pre-requisite for successful starter 56 culture development. In our previous study on key LAB in Nigerian nono samples, we identified 57 Lactobacillus (Lb.) helveticus, Limosilactobacillus fermentum, Streptococcus thermophilus and Lb. 58 delbrueckii subsp. bulgaricus to be predominant species associated with the fermentation 59 (Fagbemigun et al., 2021). To achieve starter culture development for nono fermentation, therefore, 60 suitable strains belonging to these species with appropriate technological properties should be 61 considered. An important criterion for starter strain selection is that the strains should preferentially 62 be resistant to bacteriophage infection. In the present study, therefore, we investigated Nigerian 63 nono samples for the presence of phages with lytic activity against Lb. delbrueckii subsp. bulgaricus 64 strains.

65 Currently, the databases include the genomes of nine phages infecting Lb. delbrueckii that 66 are arranged in five genetically diverse groups (i.e. a to e): i) phage LL-H and temperate phage phiJB 67 (group a) (Guo et al., 2016; Mikkonen et al., 1996), ii) phage LL-Ku (Riipinen et al., 2011), phage c5 68 (Riipinen et al., 2011), phages phiLdb, Ld3, Ld17 and Ld25A (group b) (Wang et al. 2010; Casey et al. 69 2014), iii) temperate phage JCL1032 (group c) (Riipinen et al., 2011), iv) phage 0235 (group d) 70 (Munsch-Alatossava and Alatossava, 2013) and v) phage Ldl1 (group e) (Casey et al., 2015). Among 71 these phages, c5, Ld3, Ld17, Ld25A, LL-Ku and phiLdb have recently been classified as Cequinquevirus 72 phages within the family Siphoviridae (Walker et al., 2019). Their baseplates show similarity in 73 organization and morphology to those of lactococcal P335 phages, where it has been associated with 74 strong multiple binding to the carbohydrate receptor in the host cell wall (Casey et al., 2014). In 75 addition, two group b phages (i.e. Ld17 and Ld25A) possess a genomic region comprised of two 76 putative ORFs, which resembles the KIS element (LL-K insertion sequence) of *Lb. delbrueckii* subsp. 77 lactis phage LL-K (Forsman and Alatossava, 1994). It is suggested that at least one of the genes in the

KIS element is a structural gene specifying a collar structure that may also play a role in host range
specificity (Casey et al., 2014).

In the present study, we report on the characterization of the *Lb. delbrueckii* subsp. *bulgaricus* phage PMBT4 that was isolated from Nigerian fermented *nono* with respect to its
morphology, host range and genome sequence.

83

## 84 **2. Materials and Methods**

# 85 2.1 Nono sampling and phage isolation

86 Twenty-six nono samples (fermented for 24 – 72 h) were collected from various local markets in four 87 local Nigerian states. From all samples (with high acidity, i.e. pH < 4.6), 3-5 ml were centrifuged at 88 17,500 xg (20-35 min, 4°C) and the supernatants were filtered through a 0.45  $\mu$ m Filtropur S filter 89 (Sarstedt, Germany). The double-layer plaque assay was used for phage screening, plaque 90 purification, phage titer determination, and host range analysis. Ten microliters of each filtrate were 91 spotted on MRS soft (top) agar (7.5 g l<sup>-1</sup>) (de Man, Rogosa and Sharpe (De Man et al., 1960) VWR, 92 Darmstadt, Germany), inoculated with ca. 1 x 10<sup>7</sup> colony-forming units (cfu) ml<sup>-1</sup> of a *Lb. delbrueckii* 93 subsp. bulgaricus strain isolated from nono. MRS agar was used as bottom agar. To both - MRS soft 94 agar and MRS agar - 20 mM CaCl<sub>2</sub> and 1% glycine were added. For determination of phage titers, the 95 filtered phage sample was diluted in a ten-fold dilution series and 100 µl of appropriate dilutions 96 were added to 0.3 ml of a Lb. delbrueckii subsp. bulgaricus 2-h culture (10% inoculation; final OD<sub>620 nm</sub> 97 ca. 0.5) together with 100  $\mu$ l of 100 mM CaCl<sub>2</sub>. After 10-min adsorption time at room temperature, 98 the suspension was then added to 3 ml of 50°C warm MRS soft agar (20 mM CaCl<sub>2</sub>, 1% glycine), 99 mixed and then poured on an MRS agar plate with 20 mM CaCl2, 1% glycine. Plates were incubated 100 anaerobically at 37°C for 18 h (Anaerocult jars [Merck, Darmstadt, Germany]) and AnaeroGen 3.5 L 101 sachets [Thermo Scientific, Schwerte, Germany]). Bacteriophage titers were determined as plaque-

forming units (pfu) ml<sup>-1</sup> in duplicate. Finally, phage PMBT4 was purified and concentrated by CsCl
 gradient centrifugation as described in detail elsewhere (Sambrook and Russell, 2001).

104 The bacteriophage lytic activity spectrum was tested using a panel of nine *Lb. delbrueckii* subsp.

105 *bulgaricus* strains isolated from *nono* (results not shown) and various strains from culture collections

106 (Table 1). These strains were routinely propagated in MRS broth at 37°C. Bacteriophages could only

107 be isolated from a 48-h fermented *nono* sample ("*sallah*") from the Kano State in Nigeria.

## 108 2.2 Transmission electron microscopy (TEM) analysis

109 For TEM analysis, PMBT4 purified by CsCl gradient ultracentrifugation was dialysed against SM buffer

110 (20 mM Tris-HCl pH 7.2, 10 mM NaCl, 20 mM MgSO<sub>4</sub>.7H<sub>2</sub>O). Negative staining was performed with

111 2% (w/v) uranyl acetate on ultra-thin carbon films as described previously in detail (Casey et al.,

112 2014). Transmission electron microscopy was performed at an acceleration voltage of 80 kV (Tecnai

113 10, FEI Thermo Fisher Scientific, The Netherlands), and micrographs were acquired with a MegaView

114 G2 CCD camera (Emsis, Muenster, Germany).

## 115 **2.3** Phage genome sequencing and analysis

116 For phage PMBT4 DNA isolation, a modified protocol based on the pegGOLD Bacterial DNA 117 Mini Kit (VWR) was used. Briefly, 1.5 ml of phage lysate (10<sup>11</sup> pfu ml<sup>-1</sup>) were mixed with each of 1 µl of RNAse-free DNAse (10 mg ml<sup>-1</sup>) and RNAse (10 mg ml<sup>-1</sup>) and incubated for 2 h at 37°C. The samples 118 119 were filtered through a 0.45  $\mu$ m pore-size filter and centrifuged for 2 h (15,500 xg, 4°C). After 120 discarding the supernatant, the phage pellet was resuspended in 400  $\mu$ l DNA Lysis Buffer T from the 121 DNA isolation kit. Subsequently, 2  $\mu$ l Proteinase K (20 mg ml<sup>-1</sup>) were added and the mixture was 122 incubated for 3 h at 37°C. The sample was then mixed with 200  $\mu$ l DNA Binding Buffer by inverting 123 gently, and then loaded onto a kit column. After centrifugation, the column was washed twice and 124 dried according to the manufacturer's protocol. For the elution step, 50 µl Elution Buffer heated to 125 70°C were added and incubated for 10 min at room temperature on the column.

For genome sequencing, the Nextera XT DNA Library Preparation Kit and the MiSeq Reagent Nano Kit v2 were used according to the manufacturer's instructions (Illumina, Munich, Germany) and sequencing was performed on a MiSeq (Illumina) sequencer. Assembly of generated reads to contigs was performed using Geneious 9.1.2 (Kearse et al., 2012) and SPAdes 3.11.0 (Bankevich et al., 2012) . The subsequent annotation was performed with RAST (Aziz et al., 2008).

131The proteomes of PMBT4 and related Lb. delbrueckii subsp. bulgaricus phages were132compared using all-against-all, bi-directional BLAST alignments (Altschul et al., 1990). An alignment133cut-off E-value of 0.0001, and a similarity cut-off level of at least 30% amino acid identity across 80%134of the sequence length were applied. Based on this analysis, the closest relatives of PMBT4 were135identified. The proposed functional annotations were further investigated by performing structural136homology searches via HHpred, TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and137Pfam.

#### 138 2.4 PCR-based analysis for the presence of a KIS element in PMBT4

139 To analyze whether the PMBT4 genome harbors a KIS element similar to those located in the 140 genomes of Lb. delbrueckii subsp. lactis phages LL-K (acc. no. AY739900.2 (Forsman and Alatossava, 141 1994; Riipinen et al., 2011)), Ld17 (acc. no. NC\_025420) and Ld25A (acc. no. NC\_025415), two PCR reactions with primers that bind within the putative genes for a glycerophosphoryl diester 142 143 phosphodiesterase (ORF16) and an antireceptor (ORF17) in the phage PMBT4 genome, which would 144 flank a putative KIS element if present, were used (Table 2). First, phage DNA was isolated from 1.5 145 ml of a high titer lysate (ca. 10<sup>11</sup> pfu ml<sup>-1</sup>) as described above. For amplification of genes, the 146 DreamTaq Green PCR Mastermix (Thermo Scientific) was used according to the manufacturer's 147 protocol. One  $\mu$ l (~30 ng) of the kit-purified phage DNA was mixed with primers, mastermix and 148 ddH<sub>2</sub>O and PCR-amplified using the following steps: 1) 95°C for 3 min, 2) 95°C for 30 s, 3) 54°C for 30 149 s, 4) 72°C for 1 min and 5) 72°C for 10 min. Steps 2) to 4) were repeated 35 times.

6

#### 151 3. Results and Discussion

## 152 **3.1. Phage PMBT4 morphology and host range**

153 Phage PMBT4 was assigned by transmission electron microscopy (Fig. 1) to the Siphoviridae family 154 with a long but thin, non-contractile 167 nm tail and an isometric head (diameter: 58 nm) (Table 3). 155 This phage shows a unique morphology as it has an unusually large neck passage (collar) structure 156 (width: 24 nm, Table 3 & Fig. 1), which appears larger than those of other *Lb. delbrueckii* phages 157 (Casey et al., 2014). However, this collar was only present on a minority of the phage particles 158 observed under the electron microscope (estimated at 1-5%). The majority of the phages had clearly 159 lost this structural decoration. In addition, phage PMBT4 possessed a short tail fiber (length: 16 nm), 160 protruding under the large baseplate (height: 15 nm; Fig. 1 & Table 3). Six globular appendages were 161 visible on micrographs revealing a bottom-view on the baseplate complex (Fig. 1). With this 162 morphology, phage PMBT4 particles lacking a collar resembled phage Lb. delbrueckii Ld3, while those 163 with a collar were similar to the *Lb. delbrueckii* phages Ld17 and Ld25A (Casey et al., 2014). In 164 contrast to phages Ld3 and Ld17 (Casey et al., 2014), which could only infect one or three of the Lb. 165 delbrueckii subsp. bulgaricus strains tested in this study (Table 1), phage PMBT4 exhibited a relatively 166 wide host range, as it infected nine out of the 21 (i.e. 43%) Lb. delbrueckii strains.

#### 167 3.2. Phage PMBT4 genome analysis

168 A total of 81,162 paired-end reads (2x251 bp) were generated by sequencing with MiSeq, from which 169 80,932 reads were de novo assembled into a single contig with a total length of 31,399 bp. On 170 average, the assembled genome showed more than 500-fold coverage. Annotation with RAST 171 resulted in the identification of 50 coding sequences (CDS). Phages that infect strains of the two Lb. 172 delbrueckii subspecies bulgaricus and lactis, respectively, are currently classified into five distinct 173 groups (i.e., groups a, b, c, d and e) based on DNA homology (Casey et al., 2014). Phages LL-Ku, c5 174 and Ld3, Ld17 and Ld25A are group b phages based on their sequence homology, and these were 175 isolated from dairy plants in Finland and a yoghurt production facility in France (Accolas and

Spillmann, 1979; Alatossava and Pyhtila, 1980) and more recently from whey samples from yoghurt
production facilities in Jordan and Turkey and from Gorgonzola cheese production in Italy (Casey et
al., 2014), respectively (Table 4).

Phage PMBT4 (isolated from Nigerian *nono* in this study) showed high genome sequence homology, as well as similar numbers and organization of genes with group b *Lb. delbrueckii* phages (Fig. 2). High genome homologies to phage c5 (96% identity/ 93% coverage), Ld3 (95% identity, 84% coverage), Ld25A (96% identity, 82% coverage) and Ld17 (95% identity and 83% coverage) could be detected, while only slightly lower homologies were observed when phage PMBT4 was compared to phages phiLdb (92% identity, 88% coverage) and LL-Ku (94% identity, 90% coverage).

185 These Siphoviridae phages (classified in 2018 as members of the genus Cequinquevirus by the 186 International Committee on the Taxonomy of Viruses, ICTV; (Walker et al., 2019)) possess genomes 187 with cohesive ends that vary in size from 29 to ca. 34 kbp. The largest genome among the isolates is 188 that of the virulent phage phiLdb (Wang et al., 2010) with a size of 33,996 bp. The mol% GC values 189 were very similar and ranged from 41.5 to 42.2 % (Table 4). Phage PMBT4 genome displayed a typical 190 gene organization, with genes associated with morphogenesis, replication and lysis being organized 191 within modules. The morphogenesis module starting from the portal protein-encoding gene (ORF3) 192 to the tail component protein-encoding genes (ORF14) is well conserved among the type b Lb. 193 delbrueckii phages (Casey et al., 2014) with many of the predicted proteins sharing >95% amino acid 194 identity (Fig. 2).

ORF3 is predicted to encode the portal protein; ORF4 a capsid maturation function and ORF5 is predicted to encode the major capsid protein (Fig. 2, Suppl. Table 1) with 100 % structural relatedness to that of the coliphage HK97 (PDB No. 3QPR\_D). ORFs 6-9 encode small proteins and based on their genomic location and structural relatedness (in the case of ORF7 and 8), these proteins are predicted to encode head-tail joining functions. ORF10 is predicted to encode the tail terminator protein based on structural homology searches (99.3 % probability; PDB NO. 6TE9\_F).

201 ORF11 encodes the major tail protein (96.6 % probability; PDB No. 6XGRJ). ORF12 is of unknown 202 function; however, based on its genomic location, it is likely a chaperone for the tail tape measure 203 protein. ORF13 of PMBT4 possesses two predicted transmembrane domains based on 204 transmembrane modelling predictions and bears structural similarity to the tail tape measure protein 205 of the Staphylococcus aureus phage 80 (99.5 % probability; PDB No. 6V8I BF) (Fig. 3). We propose 206 that ORF14 of PMBT4 encodes the distal tail (Dit) protein based on structural homology searches. 207 This protein is predicted to have 100 % structural similarity to the Dit protein of the Bacillus subtilis 208 phage SPP1. It is a small protein comprising 234 aa (Fig. 3) and does not possess any identifiable 209 carbohydrate binding domains and is, therefore, considered a "classical" Dit. Downstream of dit 210 ORF14, is a gene encoding a protein with unknown function (ORF15). This protein bears structural 211 similarity to a baseplate protein of the lactococcal P335 phage, TP901-1 (98.1% probability) and is, 212 therefore likely to form part of the distal tail structure of PMBT4. ORF16 is predicted to encode a 213 glycerophosphoryl diester phosphodiesterase that is believed to function as the tail associated lysin 214 (Tal), while ORF17 is predicted to encode the putative antireceptor. The glycerophosphoryl diester 215 phosphodiesterase (GDPD) was reported to be a structural component of the baseplate from phage 216 Ld17 and possesses a domain with structural similarity to GDPDs encoded by multiple bacteria (100% 217 probability) (Cornelissen et al., 2016). The putative antireceptor genes share 70-89% sequence 218 identity between the group b phages PMBT4, Ld3, Ld25A and Ld17 (Fig 2). This protein exhibits two 219 identifiable domains with similarity to tail tip proteins: at the amino terminal end, 324 aa bear 220 structural relatedness to a protein within the *Staphylococcus* phage 80 tail tip complex (99.9% 221 probability; PMD No. 6V8I\_AE) while the C-terminus contains a region with structural relatedness to 222 the coliphage T4 baseplate protein Gp10 (99.1% probability; PMD No. 2FKK A) (Fig. 3). The carboxy-223 termini of these antireceptors exhibit most variability (consistent with previous studies) (Fig. 4). The 224 repeat region starting at position 441 of the antireceptor protein was previously noted to be present 225 in phage Ld17 while absent in phage Ld25A (and PMBT4, see Figure 4), and was suggested to possibly 226 play a role in host recognition (Casey et al. 2014). This, or other differences in specific amino acid 227 residues within the binding domain (which has so far not been elucidated) may explain the unique

host range of the phages (Table 1) and the apparent broader host ranges of phages PMBT4 and Ld25
(Casey et al. 2014). Alternatively, host-factors such as CRISPR-Cas spacers that may correspond to the
genomes of phages Ld3 and Ld17 with the narrower host range, or restriction modification systems
which may recognize specific sequences that are less abundant in phages PMBT4 or phage Ld25A and
thus allow a broader host range, may be responsible for the differences in host range observed.

233 **3.3. Phage PMBT4 does not encode a KIS-element.** 

234 The genomes of phages Ld25A and Ld17 have two additional genes located between the putative 235 glycerophosphoryl diester phosphodiesterase and antireceptor genes: a gene encoding a collagen 236 repeat protein and a putative adsorption protein gene. These genes are absent in the other group b 237 Lb. delbrueckii phages, including phage PMBT4, and were previously described in Lb. delbrueckii 238 subsp. lactis phage LL-K to encode a KIS element (LL-K insertion sequence) (Forsman and Alatossava, 239 1994), which is believed to encode a neck passage structure (collar) and a putative adsorption 240 protein that might also be involved in host range determination (Casey et al., 2014). Casey et al. 241 (2014) speculated that the presence of the two KIS element genes in some bacteriophages may be 242 the result of acquisition events from other Lb. delbrueckii phages, or alternatively that they were 243 deleted from those phages who are missing these genes. Interestingly, phage PMBT4 seems to have 244 a significantly broader host range when tested for lytic activity against a variety of Lb. delbrueckii 245 strains compared to the phages Ld17 and Ld3 (Table 1), even though it lacks the KIS element genes. 246 However, our electron microscopic study of PMBT4 phage particles revealed a low amount (ca. 1-5%) 247 of phages which contained a collar structure below the head. This observation suggests that phage 248 PMBT4 possesses a KIS like element. Several attempts to isolate phage types equipped with such a 249 collar were not successful, when 50 PMBT4 phage derivatives were isolated from single plaques and 250 analysed by electron microscopy, i.e. none of the phages thus assessed showed the presence of a 251 collar (data not shown). Consequently, we searched for unassembled reads which contain the KIS 252 element genes in the raw high-throughput sequence data. Therefore, the total raw reads of phage 253 PMBT4 were mapped directly to the KIS element regions of phages Ld25A and Ld17, but no sequence

254 match was obtained (data not shown). To confirm the absence of the KIS element in the phage 255 PMBT4 genome, two PCR assays with phage PMBT4 specific primers (Table 2), that should 256 theoretically flank the KIS element genomic region between ORF16 and ORF17, were performed. In 257 detail, the following combinations were used i) primer 99fw and 100rev, which would yield a 1,753-258 bp PCR product in the presence of a KIS element but ii) only a 69-bp product in case of its absence, iii) 259 primer 99fw and 101rev, which would result in a 1,902-bp PCR product should a KIS element be 260 absent, but iv) if present, the PCR product would be 3,586 bp in size. The result of the PCR (Fig. 4) 261 showed that no KIS element could be found in the phage PMBT4 genome, as only the combination of 262 primer 99fw and 101rev resulted in a corresponding PCR product, representing the 1,902-bp DNA 263 region. Furthermore, as expected, the combination of primers 99fw and 100rev resulted in a small 264 PCR product representing the 69-bp product. Hypothetically, the presence of a defective prophage 265 on the genome of the host strain might be able to supply the collar protein to PMBT4 in trans. 266 However, when checking the chromosomal DNA of the sequenced host strain *Lb. delbrueckii* subsp. 267 bulgaricus Nono-21:328M (Cho et al., 2020), no collagen repeat-containing protein (CRP) gene or 268 adsorption protein (AdP) gene, which make up the mobile genetic element termed the KIS element, 269 could be detected on the two incomplete prophages of 10.2 kbp and 32.2 kbp identified on the 270 chromosome, respectively (results not shown). To conclude, our results suggest that the phage 271 PMBT4 collar structure observed for some PMBT4 phage particles under the electron microscope did 272 not derive from a KIS like element. The alignment of genome sequences in Fig. 2 clearly shows that 273 there is higher diversity in genes on the right half of the genome (genes located downstream of the 274 antireceptor protein gene) when compared to genes on the left half of the genome. Also, there were 275 numerous genes on the right arm of *L. delbrueckii* phage genomes which encode hypothetical 276 proteins with unknown function. Thus, it may be conceivable that there are genes in this region 277 which may encode the observed collar. However, the Blast search results of the genes (Suppl. Table 278 1) and those reported in the ORF table for phage Ld25A by Casey et al. (2014) do not allow the 279 determination of one or more candidate genes responsible for encoding such proteins.

#### 281 4. Conclusions

282 A novel *Siphoviridae* bacteriophage infecting a relatively wide range of *Lb. delbrueckii* subsp. 283 bulgaricus strains was isolated from a Nigerian fermented milk product called nono. Based on 284 genome sequencing, the phage could be assigned to the group b Lb. delbrueckii phages and the 285 genome size of 31,399 bp and the 41.6 mol% GC content compared well to the characteristics of 286 these group b bacteriophages. The close relationship to phages c5, Ld3, Ld17, Ld25A and LL-Ku based 287 on genome and morphology similarity clearly revealed that phage PMBT4 belongs to the genus 288 Cequinquevirus. The genomic analysis further revealed the presence of two genes (ORF16 and 17), 289 which encode a glycerophosphoryl diester phosphodiesterase and a putative antireceptor, 290 respectively. These genes were also found to be present in genomes of two other members of the 291 group b Lb. delbrueckii phages, i.e. Ld17 and Ld25A, where they encompass the so-called KIS 292 element. Importantly, electron microscopic studies of the phages that possess KIS elements in their 293 genomes, showed that the phages produce a neck passage structure that was hypothesized to play a 294 role in a relatively broader host range (Casey et al., 2014). To test this hypothesis that the KIS 295 element genes encode the neck passage structure also in phage PMBT4, the complete set of raw 296 sequence data from the genomic study of PMBT4 were investigated in order to determine 297 unassembled contigs in which the KIS element genes were present. Furthermore, PCR primers 298 flanking the KIS element were used to identify this DNA element in the genomic DNA pool isolated 299 from the phage lysate, which should contain phages with neck passage structure as well as phages 300 without these structures. In this study, the absence or presence of a neck passage structure could 301 not be correlated to either absence or presence of genomic sequence DNA contigs, or by a 302 differential PCR amplification targeting the KIS element genes. Therefore, the question of which open 303 reading frame(s) encode(s) a neck passage structure, and whether the loss of this structure is 304 associated with gene loss, still remains unanswered. Our results suggest that the KIS element genes 305 do not appear to be the genes associated with the neck passage structure observed for phage PMBT4

- 306 particles under TEM. Protein analyses studies may in future provide a definite answer to the
- 307 protein(s) and the gene(s) that form the biological basis for this observed structure.

- 309 Credit Authorship Contribution Statement: Conceptualization, OF, HN, FO, CF; methodology, FO, EB,
- EC, SS, JM; software, OF, EB, G-SC, EC, JM; validation, EC, EB, JM, DvS; formal analysis, OF, SS, EB, G-
- 311 SC,EC, JM; investigation, OF, EB, SS, JM; resources, CF, FO, HN, DvS; data curation, EB, G-SC, EC, SS,
- 312 JM, DvS, HN, FO, CF; writing: original draft preparation, OF, SS, HN, JM, FO, DVS, CF; writing—review
- and editing, OF, EB, EC, SS, FO, HN, JM, DvS, CF; visualization, OF, EB, SS, JM, HN, DvS, CF supervision,
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- 320 **Conflicts of Interest.** The authors declare no conflict of interest.
- 321 Data availability: The genome of phage PMBT4 has been deposited in GenBank under the accession
- no. MG913376. The version described in this paper is the version MG913376.1.

#### 323 Figure Legends

Fig. 1. Transmission electron micrographs of the *Lb. delbrueckii* subsp. *bulgaricus* phage PMBT4. The arrows indicate the central tail fiber protruding beneath the baseplate complex (1a-b, d-e). The majority of phage particles did not possess collar (neck passage) structures (1a-c). Unusually large collar structures were detected on a low number (ca. 1-5%) of PMBT4 phage particles (see triangles in 1d-e). In 1c, the six-fold symmetry of the globular subunits of a baseplate complex is visible on a PMBT4 phage particle with a folded baseplate complex.

**Fig. 2.** Genomic comparison of *Lb. delbrueckii* phage PMBT4 with other group b members. Predicted functions associated with the gene products are coded according to the colored boxes on the right. The leftward region of the genome is associated with the structural components of the phage tail and capsid (purple, blue and yellow arrows). The rightward end of the genome is associated with replication (green) and lysis (red) functions. The sequence similarity between gene products of the phages is indicated by shaded grey/black boxes with the percentage of identity (% aa) indicated by distinct grey-scale colors as indicated in the figure.

Fig. 3. HHPred outputs for the predicted tail structural proteins. Based on structural homology, it is possible to identify the (A) tail tape measure protein encoded by ORF13; (B) a "classical" or nonevolved distal tail protein encoded by ORF14; (C) a tail-associated lysin (encoded by ORF 16) with an associated glycerophosphate phosphodiester phosphodiesterase domain and; (D) the putative receptor binding/antireceptor protein (encoded by ORF17) with domains associated with tail tip and baseplate functions at the amino- and carboxy-termini, respectively.

Fig. 4 Exclusion of a KIS element in the PMBT4 genome by PCR. PCR assays were performed with
primers 99for and 100rev and 99for and 101rev, respectively, and 5 µl of each sample were
separated with an 0.8% agarose gel for 55 min at 80 V. 1: Gene Ruler 1 kb plus (Thermo Scientific); 2:
Phage PMBT4 DNA with primer 99fw and 100rev; 3: Phage PMBT4 DNA (duplicate) with primer 99fw
and 100rev; 4: negative control primer 99fw and 100rev; 5: Phage PMBT4 DNA with primer 99fw and

- 348 101rev; 6: Phage PMBT4 DNA (duplicate) with primer 99fw and 101rev; 7: negative control with
- 349 primer 99fw and 101rev; 8: Gene Ruler Mix (Thermo Scientific).



353 Fig. 1



355 Fig. 2



# 358 Fig. 3





360 Fig. 4

Table 1. Host ranges of *Lb. delbrueckii* subsp. *bulgaricus* phages PMBT4 (this study), Ld3 and Ld17
 (Casey et al., 2014).

Lb. delbrueckii subsp.	Phage	Phage	Phage	Strain source / reference
bulguricus strain	PMBT4	Ld3 ª	Ld17 ª	
Nono-21:328M	• <sup>b</sup>	-	-	Nono isolate (this study)
MBT 92063 3038a	-	-	-	Institute's strain collection <sup>c</sup>
MBT 92063-PM11	٠	-	-	Institute's strain collection
MBT 92067	-	-	-	Institute's strain collection
MBT 92068	-	-	-	Institute's strain collection
MBT 92197 Vitus	•	-	•	Institute's strain collection
MBT 92235	•	-	-	Institute's strain collection
DSMZ 20081	•	-	-	DSMZ Braunschweig (DE)
MBT 92375	-	-	-	Institute's strain collection
MBT 92376	-	-	-	Institute's strain collection
MBT 92378	-	-	-	Institute's strain collection
MBT 92059	-	-	-	Institute's strain collection
Y532-2Lb	-	-	-	Institute's strain collection
Y532-HLB-1M	-	-	-	Institute's strain collection
Y532-HLA-2M	-	-	-	Institute's strain collection
CHCC3984	•	-	•	Chr. Hansen strain collection
CHCC3606	•	-	-	Chr. Hansen strain collection
Jo1-1	٠	-	-	Institute's strain collection
Jo231-1	-	-	-	Institute's strain collection
Ldb3	-	• <sup>b</sup>	-	(Casey et al., 2014)
ldb17	•	-	● b	(Casev et al. $2014$ )

- 370 Lysis <sup>a</sup>Casey et al., (2014).

371 - no lysis

- <sup>b</sup>Strain used as host strain for phage propagation
- 373 <sup>c</sup>from Max Rubner-Institut

- **Table 2.** Primers used in this study.

Primer	binding site	Sequence 5`→3`	Тм
99fw	3`end glycerophosphoryl diester	GCAATCTTCCTCTAGCGG	58.8
	phosphodiesterase gene (ORF16)		
100rev	5`end antireceptor gene (ORF17)	CGGTAATCCCGAAAACTCGT	57.3
101rev	3`end antireceptor gene (ORF17)	CCGCTAAATAAGGTGGCATG	57.3

# **Table 3.** Dimensions of phage PMBT4.

Structure measured	Phage dimensions (nm)	Phage particles measured
Head diameter	57.6 ± 1.7	14
Collar <sup>a</sup> height	8.5 ± 0.8	16
Collar <sup>a</sup> width	24.1 ± 1.7	16
Tail length	166.7 ± 2.8	14
Tail width	$11.6 \pm 0.4$	14
Baseplate height	14.5 ± 0.8	22
Baseplate width	24.3 ± 1.6	22
Baseplate globular structures	9.4 ± 0.7	18
diameter		
Baseplate distal fiber length	15.7 ± 1.2	14

382 <sup>a</sup> When present

Table 4. Genomic features of group b *Lb. delbrueckii* phages Ld3, Ld17, Ld25A, PMBT4, c5, phiLdb and
 LL-Ku.

Characteristic	Ld3	Ld17	Ld25A	PMBT4	c5	phiLdb	LL-Ku
Length (bp)	29,616	32,975	32,799	31,399	31,841	33,996	31,080
No. of ORFs	49	50	51	50	50	59	51
GC content (mol%)	42.2	41.97	42.2	41.6	41.9	42.0	41.5
Origin	Jordan	Italy	Turkey	Nigeria	France	China	Finland
product	yoghurt	gorgonzola	yoghurt	nono	yoghurt	yoghurt	cheese whey

## 389 References

- Accolas, J., Spillmann, H., 1979. Morphology of bacteriophages of *Lactobacillus bulgaricus*, *L. lactis* and *L. helveticus* J. Appl. Microbiol. 47, 309-319.
- Alatossava, T., Pyhtila, M.J., 1980. Characterization of a new *Lactobacillus lactis* bacteriophage. IRCS
   Medical Science, Library Compendium 8(5), 297-298.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool.
   J. Mol. Biol. 215(3), 403-410.
- Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass,
  E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K.,
  Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O.,
  Vonstein, V., Wilke, A., Zagnitko, O., 2008. The RAST Server: rapid annotations using
  subsystems technology. BMC Genomics 9, 75.
- Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko,
  S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev,
  M.A., Pevzner, P.A., 2012. SPAdes: a new genome assembly algorithm and its applications to
  single-cell sequencing. J. Comput. Biol. 19(5), 455-477.
- Banwo, K., Sanni, A., Tan, H., Tian, Y., 2012. Phenotypic and genotypic characterization of lactic acid
   bacteria isolated from some Nigerian traditional fermented foods. Food Biotechnol. 26, 124 142.
- Casey, E., Mahony, J., Neve, H., Noben, J.P., Dal Bello, F., van Sinderen, D., 2015. Novel phage group
  infecting *Lactobacillus delbrueckii* subsp. *lactis*, as revealed by genomic and proteomic
  analysis of bacteriophage Ldl1. Appl. Environ. Microbiol. 81(4), 1319-1326.
- Casey, E., Mahony, J., O'Connell-Motherway, M., Bottacini, F., Cornelissen, A., Neve, H., Heller, K.J.,
  Noben, J.P., Dal Bello, F., van Sinderen, D., 2014. Molecular characterization of three *Lactobacillus delbrueckii* subsp. *bulgaricus* phages. Appl. Environ. Microbiol. 80(18), 56235635.
- Cho, G.S., Fagbemigun, O., Brinks, E., Adewumi, G.A., Oguntoyinbo, F.A., Franz, C.M.A.P., 2020. Draft
  genome sequences of *Lactobacillus helveticus*, *Lactobacillus fermentum*, and *Lactobacillus delbrueckii* strains from African fermented nono. Microbiol. Resour. Announc. 9(1) e0134219.
- 419 Cornelissen, A., Sadovskaya, I., Vinogradov, E., Blangy, S., Spinelli, S., Casey, E., Mahony, J., Noben,
  420 J.P., Dal Bello, F., Cambillau, C., van Sinderen, D., 2016. The baseplate of *Lactobacillus*421 *delbrueckii* bacteriophage Ld17 harbors a glycerophosphodiesterase. J. Biol. Chem. 291(32),
  422 16816-16827.
- 423 De Man, J.C., Rogosa, M., Sharpe, M.E., 1960. A medium for the cultivation of lactobacilli. J. Appl.
  424 Bacteriol. 23(1), 130-135.
- Fagbemigun, O., Cho, G.S., Rösch, N., Brinks, E., Schrader, K., Bockelmann, W., Oguntoyinbo, F.A.,
   Franz, C.M.A.P., 2021. Isolation and characterization of potential starter cultures from the
   Nigerian fermented milk product *nono*. Microorganisms 9 (640).
- Forsman, P., Alatossava, T., 1994. Repeated sequences and the sites of genome rearrangements in
   bacteriophages of *Lactobacillus delbrueckii* subsp. *lactis*. Arch. Virol. 137(1-2), 43-54.
- Guo, T., Zhang, C., Xin, Y., Xin, M., Kong, J., 2016. A novel chimeric prophage vB\_LdeS-phiJB from
   commercial *Lactobacillus delbrueckii* subsp. *bulgaricus*. J. Ind. Microbiol. Biotechnol. 43(5),
   681-689.
- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A.,
  Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious
  Basic: an integrated and extendable desktop software platform for the organization and
  analysis of sequence data. Bioinformatics 28(12), 1647-1649.
- 437 Mikkonen, M., Raisanen, L., Alatossava, T., 1996. The early gene region completes the nucleotide
   438 sequence of *Lactobacillus delbruecki* subsp. *lactis* phage LL-H. Gene 175(1-2), 49-57.

439 440	Munsch-Alatossava, P., Alatossava, T., 2013. The extracellular phage-host interactions involved in the bacteriophage LL-H infection of <i>Lactobacillus delbrueckii</i> ssp. <i>lactis</i> ATCC 15808. Front.
441	IVIICTODIOI. 4, 408.
44Z 1/13	consumed in most parts of Northern Nigeria. Int. J. Dairy Sci. 6, 181-189
445	Okaghue R Bankole M 1992 Use of starter cultures containing Strentococcus digcetilactis
445	Lactobacillus brevis and Saccharomyces cerevisiae for fermenting milk for production of
446	Nigerian <i>nono</i> . World J. Microbiol. Biotechnol. 8, 251-253.
447	Riipinen, K.A., Forsman, P., Alatossava, T., 2011. The genomes and comparative genomics of
448	Lactobacillus delbrueckii phages. Arch. Virol. 156(7), 1217-1233.
449	Sambrook, J., Russell., D.W., 2001. Molecular cloning: A laboratory manual. 3rd ed. Cold Springer
450	Harbor Laboratory Press, Cold Springer Harbor, N.Y.
451	Walker, P.J., Siddell, S.G., Lefkowitz, E.J., Mushegian, A.R., Dempsey, D.M., Dutilh, B.E., Harrach, B.,
452	Harrison, R.L., Hendrickson, R.C., Junglen, S., Knowles, N.J., Kropinski, A.M., Krupovic, M.,
453	Kuhn, J.H., Nibert, M., Rubino, L., Sabanadzovic, S., Simmonds, P., Varsani, A., Zerbini, F.M.,
454 455	Davison, A.J., 2019. Changes to virus taxonomy and the international Code of virus
455	Virus os (2010) Arch Virol 164(9) 2417-2429
450	Wang S Kong I Gao C Guo T Liu X 2010 Isolation and characterization of a novel virulent
458	phage (phiLdb) of Lactobacillus delbrueckii. Int. J. Food. Microbiol. 137(1), 22-27.
459	
460	
461	
461	
462	Literaturverzeichnis
463	Casey, Eoghan; Mahony, Jennifer; O'Connell-Motherway, Mary; Bottacini, Francesca;
464	Cornelissen, Anneleen; Neve, Horst et al. (2014): Molecular characterization of three Lactobacillus
465	delbrueckii subsp. bulgaricus phages. In: Applied and environmental microbiology 80 (18), S. 5623–
466	5635. DOI: 10.1128/AEM.01268-14.
467	Wang, Shaohua; Kong, Jian; Gao, Chen; Guo, Tingting; Liu, Xiaoyong (2010): Isolation and
468	characterization of a novel virulent phage (phiLdb) of Lactobacillus delbrueckii. In: International
469	<i>journal of food microbiology</i> 137 (1), S. 22–27. DOI: 10.1016/j.ijfoodmicro.2009.10.024.