The Baseplate of Lactobacillus delbrueckii Bacteriophage Ld17 Harbors a Glycerophosphodiesterase

Cornelissen, Anneleen; Sadovskaya, Irina; Vinogradov, Evgeny; Blangy, Stephanie; Spinelli, Silvia; Casey, Eoghan; Mahony, Jennifer; Noben, Jean-Paul; Dal Bello, Fabio; Cambillau, Christian & van Sinderen, Douwe (2016) The Baseplate of Lactobacillus delbrueckii Bacteriophage Ld17 Harbors a Glycerophosphodiesterase. In: JOURNAL OF BIOLOGICAL CHEMISTRY, 291(32), p. 16816-16827.

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Glycerophosphodiester phosphodiesterases (GDPD; EC 3.1.4.46) typically hydrolyze glycerophosphodiesters to sn-glycerol-3-phosphate (Gro3P) and their corresponding alcohol during pathophysiological processes in bacteria and eukaryotes. GDPD-like domains were identified in the structural particle of bacterial viruses (bacteriophages) specifically infecting Gram-positive bacteria. The GDPD of phage 17 (Ld17; GDPDLd17) representative of the group b Lactobacillus delbrueckii subsp. bulgaricus (Ldb)-infecting bacteriophages was shown to hydrolyze, besides the simple glycerophosphodiester, two complex surface-associated carbohydrates of the Ldb17 cell envelope: the Gro3P-decoration of the major surface polysaccharide D-galactan and the oligo(glycerophosphate) backbone of the partially glycosylated cell wall teichoic acid (CWTa), a minor Ldb17 cell envelope component. Degradation of CWTa occurs according to an exolytic mechanism and Gro3P-substitution is presumed to be inhibitory for GDPDLd17-activity. The presence of the GDPDLd17-homotrimer in the viral baseplate structure involved in phage-host interaction together with the dependency of native GDPD-activity, adsorption and efficiency of plating of Ca2+-ions supports a role for GDPDLd17-activity during phage adsorption and/or phage genome injection. In contrast to GDPDLd17, we could not identify any enzymatic activity for the GDPD-like domain in the neck-passage structure of phage 340, a 936-type Lactococcus lactis subsp. lactis bacteriophage.

Glycerophosphodiester phosphodiesterases (GDPD; EC 3.1.4.46) are evolutionary highly conserved proteins present in all domains of life (from bacteria to humans) (1). During degradation of the bacterial and eukaryotic cell membrane, the glycerophospholipid building blocks are first decayed by phospholipases A1 and A2 resulting in the formation of glycerophosphodiesterases. These glycerophosphodiesterases which differ based on the alcohol moiety present (e.g. choline, inositol, glycerol) are further hydrolysed by GDPDs producing the corresponding alcohol and sn-
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glycerol-3-phosphate (Gro3P). GDPDs vary in their substrate specificity, biological function and localization inside the cell. For bacteria, GDPDs, e.g. the well characterised Escherichia coli periplasmic GlpQ and cytosolic UgpQ, play an important role in glycerophospholipid metabolism, where the released alcohol moity may act as an essential bacterial growth factor and where the produced Gro3P represents a major carbon and phosphate source (2, 3). Some GDPDs are known to contribute to bacterial pathogenesis. For example, the Haemophilus influenzae GDPD, GlpQ, is a lipoprotein located in the outer membrane, and contributes to bacterial pathogenesis through choline generation from the abundant pools of degradation products of the eukaryotic cell membrane. Consequent decoration of the H. influenza cell wall with phosphorylcholine allows evasion from the host immune system through mimicry of the eukaryotic cell membrane (4). The canonical plant GDPD-encoding genes are upregulated by inorganic phosphate deprivation and have been shown to contribute to seedling growth (5) or root hair development and density (6). The seven different GDPD isoforms identified in humans display a high degree of substrate specificity, not necessarily a glycerophosphodiester, and tissue functionality. For example, Human kidney GDE2 is an osmoregulated enzyme that controls the levels of the osmoprotector glycerophosphocholine (7), while it triggers motor neuron differentiation as part of the nervous system (8).

Interestingly, various genes that encode putative GDPDs have been identified in the genomes of bacteriophages infecting Gram-positive bacteria (Clavibacter phage CMP1 (9); Staphylocoeces aureus phage K (10) and other ‘Twort’-like viruses (11-14), all group b Lb. delbrueckii phages (15-17) and Lc. lactis subsp. lactis phages 340 and 645 (18, 19)) as part of the genomic region that is involved in virion morphogenesis, although their predicted functionality has not been assessed.

The present study investigates the GDPD derived from the Lb. delbrueckii ssp bulgaricus group b bacteriophage 17 (phage Ld17) and the Lc. lactis subsp. lactis bacteriophage 340 (phage 340), and represents the first characterization of bacteriophage-encoded GDPD. Besides an in-depth biochemical and molecular analysis of the GDPD of phage Ld17 (GDPD$_{Ld17}$), we studied substrate specificity and highlight the importance of the native GDPD$_{Ld17}$ during phage infection.

EXPERIMENTAL PROCEDURES

Bacterial strains, bacteriophages and growth conditions - Lb. delbrueckii subsp. bulgaricus strains were grown at 42 °C in MRS broth (Oxoid, UK) supplemented with 1 % (wt/vol) lactose, 20 mM CaCl$_2$ and 0.5 % (wt/vol) tryptone. Escherichia coli strain MC1000 was grown aerobically at 37 °C in lysogeny broth (LB). Listeria monocytogenes strains were kindly provided by Prof. M. J. Loessner (Institute of Food, Nutrition and Health, ETH Zurich, Switzerland) (20) and cultivated aerobically in Brain Heart Infusion (BHI) medium (Oxoid) at 30 °C. Lc. lactis subsp. lactis strain IL1403, the propagation host of phage 340, and Lc. lactis subsp. cremoris NZ9000, the host strain for protein (over)expression, were cultured in GM17 broth, M17 broth (Oxoid, United Kingdom) supplemented with 0.5 % (wt/vol) D-glucose under static conditions at 30 °C. Lc. lactis subsp. cremoris NZ9000 transformants were selected on GM17 agar plates with 5 μg/ml chloramphenicol (Sigma-aldrich, United Kingdom).

Propagation of Lb. delbrueckii subsp. bulgaricus phage 17 (Ld17) (17) on its host Ldb17, and Lc. lactis phage 340 (19) on IL1403 was initiated by infecting a liquid culture of the host strain for protein (over)expression, were cultured in GM17 broth, M17 broth (Oxoid, United Kingdom) supplemented with 0.5 % (wt/vol) D-glucose under static conditions at 30 °C. Lc. lactis subsp. cremoris NZ9000 transformants were selected on GM17 agar plates with 5 μg/ml chloramphenicol (Sigma-aldrich, United Kingdom).

Adsorption assays - A 1 ml sample was taken of a bacterial suspension grown to mid-exponential phase (OD$_{600nm}$ of 0.550; ± 5*10$^7$ CFU/ml) and centrifuged (15,000 x g for 10 min). The bacterial pellet was resuspended in 900 μl MRS broth supplemented with 1 % (wt/vol)
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lactose, 0.5 % (wt/vol) tryptone and 20 mM of the relevant divalent cations, pre-warmed at 42 °C. 100 µl of 5×10⁵ PFU phages were added to generate a final multiplicity of infection (MOI) of 0.02. Following incubation at 42 °C for 10 min, the bacterial host cells (including irreversibly adsorbed phage) were removed by centrifugation (15,000 x g for 7 min). The supernatant was titrated using the double agar overlay method with MRS soft and solid agar supplemented with 20 mM CaCl₂ to determine the number of non-adsorbed or reversibly adsorbed phage.

**Cloning of GDPD and derivatives**

Purified bacteriophage genomic DNA served as template for amplification of the coding regions (of the truncated derivatives) of the (putative) glycerophosphodiesterase-encoding genes using the KOD high-fidelity DNA polymerase (Novagen, United Kingdom) and specific primer pairs (Eurofins MWG, Germany) (Table S1). Phage genomic template DNA was prepared by infecting Lc. lactis subsp. lactis IL1403 and Lb. delbrueckii subsp. bulgaricus 17 at an OD₆₀⁰ₐₚₜ of 0.15 with 340 and Ld17, respectively, followed by incubation until lysis was observed. The resulting lysate was passed through a 0.45 µm filter (Sarstedt, Germany) followed by total genomic DNA extraction (21). PCR products were digested with BamHI (Roche, Germany) and XbaI (Roche), cloned into the similarly digested expression vector pTX8048 (22) and finally ligated using T4 DNA ligase (Promega). Ligation mixtures of PCR fragments and pTX8048 were introduced into Lc. lactis subsp. cremoris NZ9000 by electroporation, and resulting transformants were selected on GM17 agar plates supplemented with 5 µg/ml chloramphenicol. The generated constructs were verified by DNA sequencing using vector-specific and internal sequencing primers (Table S1).

**Site-directed mutagenesis of amino acid residues involved in the catalytic reaction mechanism**

Site-directed mutagenesis was performed by PCR using the template plasmid pTX8048-gdpd and the respective primer couples Ld17_XXXaF and Ld17_XXXaR (the catalytic residue, X, on position xxx of the protein sequence was replaced by an alanine residue, A) (Table S1). The “overhang” primers consist of 11 nucleotides 5’ of the mutated triplet and 27 nucleotides 3’. The template DNA was extracted using a GeneJET Plasmid Miniprep Kit (Thermo Scientific) from dam⁺ E. coli MC1000 to ensure its methylation. PCR amplification was performed in 50 µl volumes with approximately 10 ng template, 1 unit KOD Hot Start DNA polymerase (EMD Chemicals, Gibbstown, NJ, USA), 1X KOD Hot Start DNA Polymerase buffer, 200 µM dNTPs, 1.5 mM MgSO₄ and 0.5 µM of the respective primers. Cycling conditions consisted of an initial polymerase activation (95 °C for 2 min) followed by 95 °C for 20 s, 60 °C for 10 s, 70 °C for 3 min for 35 cycles followed by a final extension for 7 min. PCR-generated amplicons were purified using the GeneJET PCR Purification Kit (Thermo Scientific) followed by DpnI treatment (Stratagene) at 37°C for 3 hours to restrict all methylated template plasmid. After a final purification (GeneJet PCR Purification Kit), the mutated pTX8048-gdpd was transformed into electrocompetent Lc. lactis subsp. cremoris NZ9000 and selected for on GM17-Cm³ agar plates. After plasmid extraction, the mutations were verified by DNA sequencing using vector-specific and internal sequencing primers (Table S1).

**Recombinant protein expression and purification**

Protein expression using the nisin-inducible expression system of pTX8048 provides an N-terminal protein fusion to thioredoxin to improve protein solubility and to a 6xHis-tag for protein purification purposes (22). Expression occurred at 30 °C for 3 h in Lc. lactis subsp. cremoris NZ9000 in GM17 broth supplemented with 5 µg/ml chloramphenicol after induction at OD₆₀⁰ₐₚₜ of 0.20 with 40 ng/ml nisin using Nisaplin powder (2.5 % nisin; Danisco, United Kingdom) dissolved in sterile water. Cells were harvested from suspension by centrifugation (8,700 x g for 10 min at 4 °C). The bacterial pellet was washed once in lysis buffer (10 mM Tris-HCl (pH 7.5), 0.3 M NaCl, 10 mM CaCl₂) and finally stored overnight at -20 °C.

The bacterial pellet was thawed at room temperature, resuspended in lysis buffer (10 mM Tris-HCl (pH 7.5), 0.3 M NaCl, 10 mM CaCl₂) supplemented with 30 mg/ml Henn Egg White Lysozyme (Sigma-Aldrich) and incubated at 20 °C for 30 min. The bacterial suspension was subsequently placed on ice and disrupted with glass beads in a Mini Bead Beater (BioSpec Products, Bartlesville, OK, USA) for 90 s followed by 5 min on ice for 3 cycles. Cell debris and insoluble components were discarded by centrifugation (25,000 x g for 30 min at 4 °C). Purification of the recombinant N-terminal 6xHis-tagged proteins was performed using the Ni-nitrilotriacetic acid agarose (Qiagen, United Kingdom) according to the manufacturer’s
protocol with the wash and elution buffer composed of 10 mM Tris-HCl (pH 7.5), 0.3 M NaCl, 10 mM CaCl₂ supplemented with 20 mM and 150 mM imidazole, respectively.

Protein purity was at least 95 % as estimated by SDS-PAGE. Protein concentration was estimated by the Bradford assay (23). Purified protein was dialyzed overnight against a volume of buffer at least 1000x the volume of the protein sample using the Slide-A-Lyzer® MINI Dialysis units (Pierce Biotechnology, Rockford, IL, USA).

SEC-MALS-RI analysis - Size exclusion chromatography of recombinantly purified GDPDs was performed on an Alliance HPLC 2695 system (Waters) using a 15-ml KW803 column (Shodex) and employing a phosphate-based saline (PBS) buffer system (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄ (pH 7.2)) at a flow rate of 0.5 ml/min. MALS, UV spectrophotometry, QELS, and RI analyses were executed with a MiniDawn Treos (Wyatt technology), a Photo Diode Array 2996 (Waters), a DynaPro (Wyatt technology), and an Optilab rEX (Wyatt technology), respectively (24). Mass and hydrodynamic radius were calculated with the ASTRA software (Wyatt Technology) using a dn/dc value of 0.185 mL/g.

Immu-n-EM - Purified phage preparation was dialyzed for 15 min against TGB buffer (200 mM Tris-HCl (pH 7.5), 500 mM glycine, 2 % (vol/vol) butanol) using Novagen D-tube TM dialyzer MWCO 3.5 kDa. Dialyzed phages were put over a Ni glow discharged grid for 30 min and incubated overnight (20 °C) with primary antibody solution diluted 1/90 in TGB buffer. The grid was washed in TGB and incubated for 1 h in 1:40 dilution of the secondary antibody solution (goat anti-rabbit immunoglobulin G 5 nm Gold conjugate solution). Fixation was done using 0.25 % (vol/vol) glutaraldehyde for 20 min in phosphate-based saline buffer at room temperature. The grid was washed and blotted 5 times in filtered dialyzed water. Finally, the samples were stained with 2 % phosphotungstic acid (pH 7.0) for about 30 sec and were observed using a Tecnai Spirit electron microscope operated at 120 kV and a 2,000 x 2,000-pixel CCD camera. Polyclonal antibodies against the recombinantly purified GDPDLd17 and GDPDl340 were raised in rabbits by Davids Biotechnologie GmbH (Regensburg, Germany).

**Extraction of cell wall-associated carbohydrates** - A culture flask of 800 ml fresh medium was inoculated with a 1 % bacterial overnight culture and grown overnight (± 20 h) at the appropriate temperature. Cells were harvested by centrifugation (8,700 x g for 15 min) and washed three times in 20 mM NH₄C₂H₃O₂AcOH (pH 4.7). The bacterial pellet was resuspended in 20 mM NH₄C₂H₃O₂AcOH (pH 4.7) supplemented with 4 % sodium dodecyl sulfate (SDS) and boiled in a water bath with intensive stirring for 1.5 h. After cooling the bacterial suspension to room temperature, the bacterial cells were collected by centrifugation (8,700 x g for 15 min) and the cell pellet was washed three to five times with 20 mM NH₄C₂H₃O₂AcOH (pH4.7) until no SDS-mediated foaming was observed. The pellet was resuspended in 50 ml of 5 % trichloroacetic acid and stirred for 48 h at 4 °C in the presence of acid-washed glass beads (Sigma-aldrich). Insoluble material was removed by centrifugation (8,700 x g for 15 min) and the supernatant was dialyzed against water at 4 °C. Subsequently, the dialysate was lyophilized and resuspended in 5 ml H₂O and centrifuged once more at high speed (25,700 x g for 20 min) to remove all residual insoluble material. The supernatant was collected, lyophilized and stored at room temperature.

**Purified carbohydrates** - Lipoteichoic acid (LTA) material from *Staphylococcus aureus* and *Bacillus subtilis* was purchased from Sigma-Aldrich. The LTA of *Lc. lactis* subsp. *cremoris* MG1363 was prepared by extraction with aqueous butanol according to Morath et al. (25). The extracellular teichoic acid of *L. monocytogenes* ScottA was purified as described by Brauge et al. (26). Purification of the cell-wall teichoic acid (CTTA) of the *Streptococcus epidermidis* strains 1457 and RP62A, and of the *S. aureus* strain MN8m was achieved as described previously by Sadovskaya et al. (27) and Vinogradov et al. (28), respectively. The cell wall-associated polysaccharide (PolSaccharide Pellicle, PSP), consisting of hexasaccharide repeating units linked via phosphodiesters bonds of MG1363 was purified according to Chapot-Chartier et al. (29). Extraction and purification of the individual cell wall-associated carbohydrates (surface-associated PolSaccharide 1 [sPS1], surface-associated PolSaccharide 2 [sPS2] and CTTA) of Ld17 was achieved according to Vinogradov et al. (30). CTTA was additionally repurified on a BioGel P-2 column.

**Enzyme-coupled spectrophotometric assay** - The enzymatic activity of GDPD was
quantified by measuring the production of sn-glycerol-3-phosphate (Gro3P) in a two-step coupled spectrophotometric assay. The GDPD reaction mixture (100 μl) consisted of 50 mM MOPS pH 7.5, 20 mM CaCl₂, recombinantly purified GDPD<sub>Ld17</sub>, GDPD<sub>r</sub> or derivatives, and a substrate of interest. The final concentration of the substrates was 2.5 g/l for the purified carbohydrates and the cell wall-associated carbohydrate extracts, and 1 mM for L-α-glycerophosphorylcholine (GPC, Sigma-Aldrich). After 30 min incubation at 30°C, the reaction was stopped by boiling (95 °C, 10 min) and the reaction mixture was cooled to room temperature. Buffer conditions (pH, ionic concentration, divalent cations) and temperature were adjusted if required in order to study the relevant GDPD reaction parameters.

The amount of Gro3P produced by GDPD was separately measured in a second reaction using Gro3P dehydrogenase, an oxidoreductase generating NADH in the presence of NAD⁺ and Gro3P. A volume of 80 μl GDPD reaction was added to the 900 μl Gro3P dehydrogenase reaction mixture consisting of 0.8 M hydrazine hydrate buffer (pH9) supplemented with 0.2 M glycine, 0.5 mM NAD⁺ and 10 U/ml Gro3P dehydrogenase. This assay mixture was incubated at 37 °C for 1 h until oxidation of Gro3P was complete. The reduction of NAD⁺ to NADH was measured by monitoring the absorbance increase at 340 nm with the spectrophotometer. The GDPD-activity is expressed as [ΔA<sub>340nm</sub>/([μmol GDPD*min]) with ΔA<sub>340nm</sub>=[A<sub>340nm</sub>(GDPD) – A<sub>340nm</sub>(control without GDPD)]. For each substrate, the GDPD concentration was chosen in the linear range of activity.

**HPAEC-PAD analysis** - Using the Dionex ICS-3000 system (Sunnyvale, CA), carbohydrate fractions (25 μl) were separated on a CarboPac PA1 analytical-exchange column (4 x 250 mm) with a CarboPac PA1 guard column (4 x 50 mm) and an ED40 pulsed electrochemical detector in the PAD mode (all from Dionex Corp.). Elution was performed at a constant flow-rate of 1.0 ml/min at 30 °C with eluents A, B and C consisting of 200 mM NaOH; 100 mM NaOH, 550 mM NaOAc, and Milli-Q water, respectively. The following linear gradient of NaOAc was used with 100 mM NaOH: 0 - 50 min, 0 mM; 50 - 51 min, 16 mM; 51 - 56 min, 100 mM; 56 - 61 min, 0 mM. The CHROMELEON software Ver. 670 (Dionex Corp.) enabled the evaluation of the chromatograms. Enzymatic reactions consisting of 1 μM recombinantly purified GDPD and 2.5 g/l purified carbohydrates or cell wall-associated carbohydrate extracts were performed at 30 °C for 2 h in a 50 mM MOPS (pH 7.5), 20 mM CaCl₂ buffer system. As positive control for the detection of Gro3P, the aforementioned reaction was performed with 1 mM GPC and 1 μM GDPD<sub>Ld17</sub>. Negative controls used for each HPAEC run were (i) buffer control: 50 mM MOPS (pH 7.5), 20 mM CaCl₂ buffer, (ii) (Negative control for the enzyme): 50 mM MOPS (pH 7.5), 20 mM CaCl₂ buffer plus 1 μM GDPD, (iii) (Negative control for GPC): 50 mM MOPS (pH 7.5), 20 mM CaCl₂ buffer plus 1 mM GPC, (iv) (Negative control for each carbohydrate) 50 mM MOPS (pH 7.5), 20 mM CaCl₂ buffer plus the relevant carbohydrate substrate.

**NMR analysis of Ldb17 sPS2 after GDPD<sub>Ld17</sub> treatment** - Solutions of purified Ldb17 sPS2 (2 g/l, solution A) and GDPD<sub>Ld17</sub> (10 μM, solution B) were prepared in a 50 mM MOPS (pH7.5), 20 mM CaCl₂ buffer system. 0.5 ml of solution A and 0.1 ml solution B were mixed, and the final volume adjusted to 1 ml with MOPS-CaCl₂ buffer (final concentrations -1 mg/ml of purified Ldb17 sPS2 and 1 μM GDPD<sub>Ld17</sub>). In the control experiment, buffer was used instead of GDPD<sub>Ld17</sub> solution. Reaction mixtures were incubated for 2 h at 30 °C. A 100 μl TCA solution (50 %) was added to precipitate the proteins, and the mixtures were fractionated on a Sephadex G-50 column. Elution profiles of two samples were identical, indicating the absence of depolymerization. Fractions corresponding to polysaccharides were collected, lyophilized and repurified on a Sephadex G-50 to remove traces of MOPS, and analyzed by NMR spectroscopy as described in Vinogradov et al. (30). In a separate experiment, the phosphate content in the GDPD<sub>Ld17</sub>-treated and untreated sPS2 sample was assessed according to Chen et al. (31).

**Exohydrolytic activity of GDPD<sub>Ld17</sub> on CWTA** - The reaction mixture constituted of 1 ml purified CWTA (1 g/l) of *Staphylococcus epidermidis* strains 1457 and 5 (32), 1 μM GDPD<sub>Ld17</sub> and 50 mM MOPS (pH 7.5), 20 mM CaCl₂ buffer. After incubation for 2 h at 30 °C, the chromatographic profile of the reaction mixture and the untreated purified CWTAs were analyzed on a Sephadex G-50 gel filtration column as described in Vinogradov et al. (30).
RESULTS

The presence of a GDPD domain in structural phage proteins with distinct topologies - The structural module of the genomes of all known group b Lb. delbrueckii-infesting phages, and of the Lc. lactis subsp. lactis-infesting phages 340 and 645, contains a gene that encodes a protein containing a GDPD-(like) domain. Because of high protein identity of these putative GDPD-containing proteins within the group b Ldb-infesting phages (93 to 95 % identity) and between the two Lc. lactis ssp. lactis-infesting phages (phages 340 and 645; 99 % identity), the GDPD of phage Ld17 (designated here as GDPDLd17) and 340 (named GDPD340) were selected as representatives of the respective phage groups.

Bioinformatic analysis revealed an apparent tripartite domain structure in both GDPDs (Fig. 1A): (i) an N-terminal domain that is presumably required for physical association with the phage particle, (ii) a putative carbohydrate-binding domain, and (iii) the GDPD-(like) domain, with the latter two domains displaying an inverse arrangement in GDPDLd17 and GDPD340 (Fig. 1A).

NCBI Conserved Domain Detection analysis (33) identified a DUF2479 domain (residues 23-167; 9.76E-06) within the N-terminus of GDPD340, a typical feature of the neck-passage structure (NPS) of lactococcal 936-type phages to which phage 340 belongs (34). Transmission electron micrographs of immunogold-labelled GDPD340 confirm the presence of GDPD340 as part of the phage 340 NPS (Fig. 2B). No conserved domains were detected in the N-terminus of GDPDLd17; while immuno-EM using anti-GDPDLd17 antibodies revealed that the GDPD protein of Ld17 is associated with the baseplate of phage Ld17 (Fig. 2A).

While a conserved carbohydrate-binding domain (CBM_4_9; residues 664-788; 9.76E-03) is present in the C-terminus of GDPDLd17, we hypothesize that a carbohydrate-binding function is present in the central domain of GDPD340 based on the identification of a ± 124 amino acid repeating unit suggesting interaction with a (repeated) component of the bacterial cell envelope, using the Trust server (35).

Sequence similarity between GDPDLd17 and GDPD340 is restricted to their GDPD-(like) domains with 24.8 % amino acid identity of the GDPD-like domain of GDPD340 compared to this of GDPDLd17. The GDPD-domain (residues 378-613) of GDPDLd17 belongs to the large family of the canonical prokaryotic and eukaryotic GDPDs (EC 3.1.4.46), represented by the well-characterized cytoplasmic E. coli protein UgpQ. ClustalW alignment (Fig. 1B) highlights in the GDPD-domain of GDPDLd17 the presence of five conserved amino acid residues - two catalytic histidine residues (H381 and H421 in GDPDLd17) and three residues (E408, D410 and E491) for binding a divalent cation – which are believed to be involved in the catalytic metal ion-dependent acid-base reaction mechanism to hydrolyse glycerophosphodiester linkages (36). The GDPD-like domain (residues 635-795) of GDPD340 belongs to a subfamily of uncharacterized bacterial GDPDs (cd08582; 7.40E-15); curiously, ClustalW-alignment identified just two (H669 and E737) of the five conserved residues that are associated with the typical GDPD-mediated reaction mechanism (Fig. 1B).

Structural characteristics - Consistent with the immunogold electron microscopy findings (Fig. 2), mass spectrometric analyses of Ld17 (17) and 340 (Table S2) virions indicates the presence of the full-length GDPDs as part of the structural phage particle. Full-length GDPDLd17 and GDPD340 were recombinantly expressed and purified using the Lc. lactis NZ9000 – pTX8048 expression system (22). Mass calculations using SEC-MALS-RI analyses of the purified, recombinant GDPDLd17 and GDPD340 proteins revealed the presence of a complex with an estimated mass of 325 kDa and 326 kDa, respectively, which corresponds to a homotrimeric native conformation (theoretical molecular weight of 111.9 kDa and 110.7 kDa for GDPDLd17 and GDPD340 monomers, respectively) (Fig. 3A). Moreover, GDPDLd17 appears to form an SDS-resistant homotrimer at room temperature (Fig. 3B). An estimated molecular mass of ~335 kDa, consistent with a homotrimer, was observed when unheated samples of GDPDLd17 were loaded on a 7 % (w/v) SDS-PAGE gel, whereas a band consistent with the theoretical molecular weight of the monomer (111.9 kDa) was observed when the protein sample was boiled for 5 min prior loading on the SDS-PAGE gel. The GDPD340 protein does not display this SDS-resistant characteristic at room temperature and appears to disassemble into its monomers with or without a heating step prior to or upon electrophoresis.

Enzymatic activity and substrate preference of GDPDLd17 - During metabolism or pathogenesis, bacterial GDPDs are known to hydrolyze deacylated phospholipids, called glycerophosphodiesterases, into the corresponding
alcohol and sn-glycerol-3-phosphate (Gro3P). Therefore, we analysed in the first instance the ability of the bacteriophage-encoded GDPDs to degrade glycerophosphorylcholine (GPC), a glycerophosphodiester, with HPAEC-PAD analysis in parallel with an enzyme-coupled spectrophotometric assay quantifying the formation of Gro3P. The specific GDPD-activity of GDPD$_{Ld17}$, expressed as [ΔA$_{340nm}$/ (μmol GDPD*min)], using 2.5 mM GPC substrate after 30 min incubation at 30 °C was 7466.67 ± 108.44 (Fig. S1A). In parallel, the HPAEC-PAD chromatogram showed one additional peak at 42 min after GDPD$_{Ld17}$ treatment of the GPC substrate which we attribute to the formation of Gro3P (data not shown).

As the bacterial GDPDs perform a second step in cell membrane degradation subsequent to deacetylation of the glycerophospholipids by phospholipase A, we presumed that these glycerophosphodiesters are not natural substrates of the bacteriophage-encoded GDPD, but that this activity is serendipitous. Since bacteriophage-encoded GDPDs, being a structural component of the virion, are most likely to interact with phosphodiester-containing substrates (e.g., teichoic acids (37), or other surface-associated carbohydrates (29)) in the host cell envelope, we implemented an extraction procedure to concentrate cell wall-associated carbohydrates (CWaC), excluding peptidoglycan.

HPAEC-PAD analysis of the CWaC-extract of Ldb17, the bacterial host of phage Ld17, displayed an extra peak at 42 min following GDPD$_{Ld17}$-treatment compared to the negative buffer control (data not shown). This peak corresponds to the Gro3P peak liberated in the presence of the GPC substrate. Using the enzyme-coupled spectrophotometric assay specific for the detection of Gro3P, the GDPD$_{Ld17}$-activity on the Ldb17 CWaC-extract was quantified, 2071.11 ± 37.91 [ΔA$_{340nm}$/ (μmol GDPD*min)]. These results imply the presence of carbohydrate substrates in the Ldb17 cell envelope which following GDPD$_{Ld17}$-hydrolysis generate an identical reaction product as that during GPC hydrolysis, namely Gro3P.

We have recently identified two carbohydrates associated with the Ldb17 cell surface which contain Gro3P: a Gro3P-substituted linear D-galactan, sPS2, with the following repeating unit (α-Gro3P-1→6)-3-β-Gal/3-α-Galp-2-β-Galp-6-β-Gal/3-β-Galp-) and short chain cell wall teichoic acid (CWTA) structures with a partially glycosylated poly(glycerophosphate) backbone (30). Using the enzyme-coupled spectrophotometric assay a GDPD$_{Ld17}$-activity of 31.56 ± 0.77 and 873.33 ± 13.33 [ΔA$_{340nm}$/ (μmol GDPD*min)] was identified on the CWTA and the Gro3P-decorated D-galactan of Ldb17, respectively (Table 1). NMR analysis of GDPD$_{Ld17}$-treated sPS2 showed the absence of all signals of Gro3P (Fig. 4). While native sPS2 contained approximately 0.7 μM/mg of phosphate, the phosphate content of the GDPD$_{Ld17}$-treated sPS2 was below the level of detection. HPAEC-PAD (Table 1) further confirm the cleavage of Gro3P from these purified carbohydrates after exposure to GDPD$_{Ld17}$.

To gain further insight in the substrate specificity of GDPD$_{Ld17}$, a spectrum of purified carbohydrates after treatment with GDPD$_{Ld17}$ (in parallel with a buffer control) was analyzed with HPAEC-PAD. According to HPAEC-PAD analyses (Table 1), the sole end product of GDPD$_{Ld17}$-hydrolysis is Gro3P which is removed from the polyglycerophosphate CWTA backbone, the Gro3P-decorated linear D-galactan and the bacterial GPC. The single Gro3P peak observed in the HPAEC-PAD chromatograms of all poly(glycerophosphate) CWTA exposed to GDPD$_{Ld17}$ suggests exolytic activity for GDPD$_{Ld17}$, whereby Gro3P units are released one by one from the CWTA ends. This is consistent with the obtained results from gel filtration chromatography of the long poly(glycerophosphate) CWTA of two S. epidermidis strains, 1457 and 5 (Fig. S2) which show no appearance of smaller oligo(glycerophosphate) after exposure to GDPD$_{Ld17}$. Furthermore, the absence of peaks other than that corresponding to Gro3P and the correlation between the observed Gro3P peak volume in the HPAEC-PAD chromatograms and the Gro3P-quantification by this enzyme-coupled spectrophotometric assay suggests that decorated, typically by glycosylation or D-alanylation for CWTA, Gro3P units in the CWTA backbone are not removed. This indicates that GDPD$_{Ld17}$-mediated degradation of CWTA is terminated at a substituted Gro3P unit. This notion is consistent with the low GDPD$_{Ld17}$-activity (11.11 ± 0.77) observed for S. epidermidis RP62A CWTA (27), which has a high degree of substitution compared to S. aureus NM8m (28) (137.33 ± 1.33) and B. subtilis 168 (38) (56.67 ± 3.45) (Table 1). Other phosphodiester bonds – i.e. those that connect hexasaccharide repeats in the lactococcal pellicle of MG1363 (29) or those present in the...
polyribitolphosphate TA of *Bacillus subtilis* W23 (39) – are insensitive to GDPD<sub>Ld17</sub>-hydrolysis, thus indicating a requirement for glycerol adjacent the phosphodiester bond (Table 1). However, lipoteichoic acid (LTA) is not degraded by GDPD<sub>Ld17</sub>, despite the fact that CWTA and LTA share a similar (1→3) poly(glycerol)/phosphate backbone. It should be noted that CWTA possesses a Gro3P repeating unit, while the repeating unit (and thus the unit available for GDPD<sub>Ld17</sub> at the end) of the LTA backbone is Gro1P which has an opposite stereochemistry on C2 of the glycerol unit compared with Gro3P. The absence of any degradation in the HPAEC-PAD chromatogram of purified LTAs following GDPD<sub>Ld17</sub> treatment, indicates a strict preference for the Gro3P configuration.

**Biochemical and structural requirements for GDPD<sub>Ld17</sub>-activity** - The optimal biochemical characteristics for GDPD<sub>Ld17</sub>-activity were assayed on Ld17 CWaC-extract (3 µg/µl) in the linear range of enzymatic activity using the enzyme-coupled spectrophotometric assay (See Supplementary information for a detailed description, Fig. S3). The highest GDPD<sub>Ld17</sub>-activity is observed in the temperature range of 30 to 42°C at pH8 in the presence of 10 mM Ca<sup>2+</sup>. While Mg<sup>2+</sup> gradually inhibits GDPD<sub>Ld17</sub>-activity upon increasing concentration with 25 % relative activity at 100 mM MgCl<sub>2</sub>, other divalent cations (Mn<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, Cd<sup>2+</sup> and Zn<sup>2+</sup>) display a stronger inhibitory effect with complete absence of enzymatic activity at 5 mM.

Since most bacterial GDPDs in the NCBI database are of similar size (30–40 kDa) as the GDPD-domain of GDPD<sub>Ld17</sub>, the individual central GDPD-domain alone (48.8 kDa), or this domain in combination with the putative carbohydrate-binding domain (73.8 kDa) were recombinantly expressed using the pTX8048 lactococcal expression vector and their enzymatic activity evaluated using the enzyme-coupled spectrophotometric assay on the Ldb17 CWaC-extract and GPC. Both truncated derivatives had lost more than 98 % activity in comparison to the full-length GDPD<sub>Ld17</sub> protein (Fig. S1A) as well as the ability to form homotrimers resistant to SDS at room temperature (Fig. S1B). As trimerization of the truncated proteins was not analyzed, one may argue that the ability to form homotrimers was already lost upon truncation. Nevertheless, loss of (SDS-resistant) trimerization upon truncation suggests that protein folding was affected, which is consistent with the near complete loss of enzymatic activity. Individual site-directed mutagenesis of each of the five conserved residues (H381, E408, D410, H423 and E491; Fig. 1B) of GDPD<sub>Ld17</sub> putatively involved in a typical GDPD-reaction mechanism reduced the total activity to below 3 %, both on the glycerophosphodiester (GPC) substrate and on the (assumed) native substrate (Ld17 CWaC-extract) for each of the five generated mutant proteins. SDS-PAGE gel electrophoresis without prior boiling of the protein sample indicates that these five mutated proteins are still able to form SDS-resistant homotrimers (Fig. S1B), implying no major conformational changes of the mutagenized proteins and affirming the involvement of the five conserved residues within GDPD<sub>Ld17</sub> in the hydrolysis of the typical glycerophosphodiester and of the native Ld17 carbohydrates.

**Absence of detectable enzymatic activity for GDPD<sub>340</sub>** - The HPAEC-PAD chromatograms did not show any activity of GDPD<sub>340</sub> on GPC and on CWaC-extracts of the phage 340 host, IL1403, or of other lactococcal and *Lactobacillus* strains (data not shown). Also the enzyme-coupled spectrophotometric assay, with higher sensitivity than HPAEC-PAD for Gro3P detection could not detect any GDPD-activity. This absence of enzymatic activity for GDPD<sub>340</sub> suggests that this protein does not possess GDPD-activity, which may have been caused by mutations that have altered three out of the five conserved residues implicated in the GDPD catalytic reaction mechanism (Fig. 1B).

**GDPD-activity and phage Ld17 characteristics** - The cation Ca<sup>2+</sup> is essential for enzymatic activity of the recombinantly expressed GDPD<sub>Ld17</sub>, while other divalent cations inhibit enzymatic activity (see above). In order to evaluate the importance of the native GDPD-activity in the baseplate of the Ld17 virion for the phage infection process, different phage characteristics (native GDPD-activity, adsorption and EOP) were assessed in the presence of a diverse set of 20 mM divalent cations (Table 2). Similar to the recombinantly expressed GDPD<sub>Ld17</sub>, the native GDPD-activity of the Ld17 virion is stimulated by the addition of Ca<sup>2+</sup> ions (relative activity of 127 %), while the other divalent cations have an inhibitory effect. Mg<sup>2+</sup> is again the less potent inhibitor with 33 % activity remaining compared to the complete absence of GDPD-activity after addition of any other inhibiting divalent cation. In parallel with the
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GDPD-activity, the adsorption and the relative efficiency of plating of phage Ld17 on the host Ld17 are optimal with values of 91 % and 1.00, respectively, in the presence of 20 mM CaCl2. Adsorption and plaque formation are less efficient in the present of the other inhibiting divalent cations with only 71 % adsorption and a relative EOP of 0.57 for Mg2+.

Further evaluation of the enzymatic activity of the recombinantly expressed GDPDld17 on CWaC-extracts of a diverse set of 12 Lb. delbrueckii subsp bulgaricus strains (Table 3), six sensitive (Ldb9, 10, 2, 14, 41, 40) and six resistant (Ldb17, 36, 22, 34, 35, 66) for phage Ld17 infection (17), indicates that all strains have carbohydrate structures associated with the cell envelope sensitive for GDPD-activity. The variation within the GDPD-activity is further independent from the phage Ld17-sensitivity of the Lb. delbrueckii host.

DISCUSSION

The GDP of bacteriophage Ld17 displays the characteristics of the canonical GDPD-activity as identified in literature (1): enzymatic activity requires (i) divalent cation(s), and (ii) a conserved set of five amino acids for a metal ion-dependent, acid-base reaction mechanism to hydrolyse a (iii) glycerophosphodiester substrate to Gro3P and an alcohol. GDPDld17 solely requires Ca2+ for activity, while other divalent cations are inhibitory for activity. This is probably a reflection of an adaptation to the high Ca2+-levels present in the dairy environment from which the Ld17 phage was isolated (17). Also the optimal temperature range (30 to 42 °C) for activity reflects the preferred growth conditions of the Lactobacillus host. Besides degradation of the simple glycerophosphodiester GPC, GDPDld17 also elicits activity towards more complex substrates, the Gro3P-decorated of the Ld17 D-galactan polysaccharide and the poly(glycerophosphate) backbone of CWTA, in all cases releasing Gro3P. Degradation of CWTA occurs according to an exo-mechanism and substitution of the CWTA backbone is presumed to be inhibitory for GDPDld17-activity. Although GDPD-hydrolysis of more complex substrates has not been identified before for the canonical GDPDs, the GDPDld17-activity is still in congruence with the recognition of the glycerophospho-moiety by the canonical GDPDs and the formation of Gro3P end-product (36, 40): the three conserved residues (E, D and E) in the catalytic groove coordinate a divalent cation which stabilizes the cyclic phosphate intermediate formed by glycerol and phosphate during a two-step reaction mechanism with the two catalytic histidine residues acting as general acid and base. The involvement of the glycerol unit in the catalytic reaction mechanism explains the absence of any hydrolyzing activity by GDPDld17 on carbohydrates with phosphodiester bonds not adjacent a glycerol unit, the Lc. lactis MG1363 pellicle and poly(ribitol)phosphate TA. Moreover, the reaction mechanism is specific for sn-glycerol-3-phosphate - the repeat unit of the CWTA backbone - while the stereoisomer sn-glycerol-1-phosphate – the repeat unit of the poly(glycerolphosphate) backbone of lipoteichoic acid is not recognized by GDPDld17.

CWTA degradation by bacterial enzymes has so far only been described in, now vintage, research articles by Kusser and Fiedler (41-43). During sporulation, B. subtilis strain Marburg produces a TA-degrading enzyme with absolute substrate specificity for the endogenous α-glucosylated glycerol TA of the cell, producing the α-D-glucose 1→2(sn)glycerol-3-phosphate according to an exo-mechanism (41, 42). Phosphate-starved Bacillus pumilus DSM27 cells produce a TA-degrading enzyme which produces Gro3P using unsubstituted TA of the polyglycerophosphate type as substrate, whereas the enzyme is unable to remove substituted glycerophosphate. Although both bacterial TA-degrading enzymes display GDPD-like activity, the question remains if the canonical bacterial GDPDs also display this teichoic-acid degrading activity. Further research of the bacterial GDPDs towards substrate specificity is, as such, required.

Immuno-EM analysis showed that GDPDld17 is part of the baseplate of phage Ld17. GDPDld17-activity removes the Gro3P-decoration of the D-galactan polysaccharide, the major charged carbohydrate associated with the Ldb17 cell wall and can further degrade the short chain partially glycosylated oligo(glycerophosphate) CWTA which is a minor cell envelope component (30). Within bacteriophages a very diverse set of enzymatic activities within baseplate-associated proteins, tail spikes or fibers, have been identified which interact with a component of or closely associated with the bacterial cell envelope. These virion-associated enzymes (partially and locally) degrade the (corresponding) bacterial cell envelope component to accomplish accessibility towards the underlying primary bacterial receptor to which the bacteriophage will irreversibly
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adsorb. To this group belong structural enzymes degrading lipopolysaccharide (44-46), exopolysaccharide (47-49), and capsule (50-52) structures and a small set of capsule deacetylases (53-54). Furthermore, peptidoglycan-degrading enzymes associated with the tail tip of the virion ensures injection of the phage genomic DNA in the bacterial cell (55-59). Within the baseplate-encoding region of phage Ld17, no peptidoglycan-degrading activity was predicted and since the carbohydrate substrates of GDPD<sub>Ld17</sub> are newly identified, it is difficult to infer if GDPD-activity of phage Ld17 is involved in rendering accessibility towards the bacterial receptor and/or injection of the phage genomic DNA in the cytosol. Moreover, the Ca<sup>2+</sup>-requirement for optimal GDPD<sub>Ld17</sub>-activity and for efficient phage Ld17 adsorption and plaque formation, suggests a general adaptation of the enzymatic activities of phage Ld17 and the different steps of its infection cycle to a Ca<sup>2+</sup>-rich environment or a dependency of adsorption and/or genomic DNA injection on GDPD<sub>Ld17</sub>-activity resulting in a more efficient infection cycle.

Group b Lb. delbrueckii-infecting phages, representing the most important genotype infecting Lb. delbrueckii possess a structural GDPD which shows 93-95 % amino acid identity to GDPD<sub>Ld17</sub> and all have a full set of the conserved five amino acids involved in the canonical GDPD-activity. Also all tested CWaC-extracts of Lb. delbrueckii subsp. bulgaricus strains were shown to be highly sensitive for GDPD-activity (Table 3). As such, further research unraveling the precise role of the GDPD during the infection process of group b Lb. delbrueckii phages will provide us with further insights for new strategies to prevent lysis of starter cultures during dairy fermentation. The absence of any detectable LTA and only limited amounts of CWTA (30), which is susceptible to GDPD-hydrolysis, further suggest adsorption to a cell wall component different from TA, whereas most bacteriophages infecting Gram-positive bacteria (e.g. Staphylococcus species (60, 61), Bacillus subtilis (62-64), L. monocytogenes (20, 65) and group a Lb. delbrueckii phage LL-H (66)) adsorb to TA-structures.

We were not able to identify any enzymatic activity for GDPD<sub>340</sub>. In comparison with the canonical GDPDs, GDPD<sub>340</sub> seems to have lost three out of five amino acid residues essential for canonical GDPD-activity. GDPD<sub>340</sub> is part of the NPS of phage 340, a 936-type phage. The lactococcal 936-type phages are a well-studied phage group because of their importance for the dairy industry, often (negatively) influencing dairy fermentation processes. The ‘older’ 936-type phage isolates have no NPS (e.g. sk1 and p2) or a truncated NPS without any enzymatic domain (e.g. bIL140, P008). More recent phage isolates from the dairy environment frequently possess a large NPS with a potential enzymatic domain, a GDPD-like domain (phages 340 and 645; 18, 19) or a SNGH-hydrolase domain (67, 68). These ‘novel’ enzymatic activities within the 936-type NPS are probably beneficial, but not essential, for bacteriophage infection in the dairy environment. It therefore appears that such enzymatic activity merely constitutes an accessory function that can be lost without loss of phage viability, as can hypothesized for GDPD<sub>340</sub>. Nonetheless, GDPD<sub>340</sub> may on the other hand represent a non-canonical GDPD with activity different from the typical hydrolysis of glycerophosphodiester and the reaction conditions used in this study may have been unsuitable for detection of this enzymatic activity. For example, the mammalian GDE4 and GDE7 degrade glycerolysolipids (1), the GDPD of Mycoplasma hyorhinis displays besides a typical GDPD activity also nonspecific phospholipase C activity (69). Furthermore, Simockova et al. (70) identified phospholipase C-activity which controls the phosphatidylglycerol content of the cell membranes in the bioinformatically annotated GDPD-lysote protein Pge1p of Saccharomyces cerevisiae.
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**Author contributions:** AC, DvS and JM conceived the idea for the project. AC conducted most of the experiments, analyzed the results, and wrote the paper. AC, DvS, JM, IS, and EV were involved in interpretation of the results. EC and FDB have set up the initial scientific frame for the project. IS and EV purified the carbohydrates, conducted NMR-analysis and Sephadex G-50 gel filtration. JPN performed mass spectrometry. CC and SB performed SEC-MALS-RI analysis. CC and SS created the immuno-EM images. All authors reviewed the results and approved the final version of the manuscript.
REFERENCES

1. Corda, D., Mosca, M.G., Ohshima, N., Grauso, L., Yanaka, N. and Mariggiò, S. (2014) The emerging physiological roles of the glycerophosphodiesterase family. FEBS J. 281, 998–1016

2. Larson, T.J., Ehrmann, M., Boos, W., and Nakata, A. (1989) Periplasmic glycerophosphodiesterases phosphodiesterase of Escherichia coli, a new enzyme of the glp regulon. J. Biol. Chem. 258, 5428-5432

3. Tommassen, J., Eigelmeier, K., Cole, S.T., Overduin, P., Larson, T.J., and Boos, W. (1991) Characterization of two genes, glpQ and upgQ, encoding glycerophosphoryl diester phosphodiesterases of Escherichia coli. Mol. Gen. Genet. 226, 321-327

4. Ahren, I.L., Janson, H., Forsgren, A., and Riesbeck, K. (2001) Protein D expression promotes the adherence and internalization of non-typeable Haemophilus influenzae into human monocytic cells. Microb. Pathog. 31, 151-158

5. Cheng, L., Bucciarelli, B., Liu, J., Zinn, K., Miller, S., Patton-Vogt, J., Allan, D., Shen, J., and Vance, C.P. (2011a) White lupin cluster root acclimation to phosphorus deficiency and root hair development involve unique glycerophosphodiester phosphodiesterases. Plant Physiol. 156, 1131-1148

6. Cheng, Y., Zhou, W., El Sheery, N.I., Peters, C., Li, M., Wang, X., and Huang, J. (2011b) Characterization of the Arabidopsis glycerophosphodiesterase (GDPD) family reveals a role of the plastid-localized AtGDPD1 in maintaining cellular phosphate homeostasis under phosphate starvation. Plant J. 66, 781-795

7. Gallazzini, M., Ferraris, J.D. and Burg, M.B. (2008) GDPD5 is a glycerophosphocholine phosphodiesterase that osmotically regulates the osmoprotective organic osmolyte GPC. Proc. Natl. Acad. Sci. USA 105, 11026-11031

8. Rao, M., and Sockanathan, S. (2005) Transmembrane protein GDE2 induces motor neuron differentiation in vivo. Science, 309, 2212-2215

9. Wittmann, J., Gartemann, K.H., Eichenlaub, R., and Dreiseitlmann, B. (2011) Genomic and molecular analysis of phage CMP1 from Clavibacter michiganensis subspecies michiganensis. Bacteriophage 1, 6-14

10. Gill, J. J. (2014) Revised Genome Sequence of Staphylococcus aureus Bacteriophage K. Genome Announc. 10.1128/genomeA.0173-13

11. Kwan, T., Liu, J., DuBow, M., Gros, P., and Pelletier, J. (2005) The complete genomes and proteomes of 27 Staphylococcus aureus bacteriophages. Proc. Natl. Acad. Sci. 102, 5174-5179

12. Vandersteegen, K., Mattheus, W., Ceyssens, P-J., Bilocq, F., De Vos, D., Pirnay, J.P., Noben, J.P., Merabishvili, M., Lipinska, U., Hermans, K., and Lavigne, R. (2011) Microbiological and molecular assessment of bacteriophage ISP for the control of Staphylococcus aureus. PLoS One. 10.1371/journal.pone.0024418

13. Gu, J., Liu, X., Lu, K., Li, Y., Song, J., Lei, L., Sun, C., Feng, X., Du, C., Yu, H., Yang, Y., and Han, W. (2012) Complete Genome Sequence of Staphylococcus aureus Bacteriophage GH15. J Virol. 86, 8914–8915

14. Cui, Z., Song, Z., Wang, Y., Zeng, L., Shen, W., Wang, Z., Li, Q., He, P., Qin, J., and Guo, X. (2012) Complete genome sequence of wide-host-range Staphylococcus aureus phage JD007. J Virol. 86,13880-13881

15. Wang S, Kong J, Gao C, Guo T, and Liu X. (2010) Isolation and characterization of a novel virulent phage (phiLdb) of Lactobacillus delbrueckii. Int. J. Food Microbiol. 137, 22–27

16. Riipinen K-A, Forsman P, and Alatossava T. (2011) The genomes and comparative genomics of Lactobacillus delbrueckii phages. Arch. Virol. 156, 1217–1233

17. Casey E, Mahony J, O’Connell-Motherway M, Bottacini F, Cornelissen A, Neve H, Heller KJ, Noben JP, Dal Bello F, and van Sinderen D. (2014) Molecular characterization of three Lactobacillus delbrueckii subsp. bulgaricus phages. Appl. Environ. Microbiol. 80, 5623-5635

18. Dupont, K., Janzen, T., Vogensen, F.K., Josephsen, J., and Stuer-Lauridsen, B. (2004) Identification of Lactococcus lactis genes required for bacteriophage adsorption. Appl. Environ. Microbiol. 70, 5825–5832

19. Mahony, J., Kot, W., Murphy, J., Ainsworth, S., Neve, H., Hansen, L.H., Heller, K.J. Sørensen, S.J. Hammer, K., Cambillau, C., Vogensen, F.K., and van Sinderen, D. (2013) Investigation of the
relationship between lactococcal host cell wall polysaccharide genotype and 936 phage receptor binding protein phylogeny. *Appl. Environ. Microbiol.* 79, 4385–4392.

20. Eugster, M.R., and Loessner, M.J. (2012) Wall teichoic acids restrict access of bacteriophage endolysin Ply118, Ply511, and PlyP40 cell wall binding domains to the *Listeria monocytogenes* peptidoglycan. *J. Bacteriol.* 194, 6498-6506.

21. Sambrook, J., and Russell, D.W. (2001) *Molecular cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

22. Douillard, F.P., O'Connell-Motherway, M., Cambillau, C., and van Sinderen D. (2011) Expanding the molecular toolbox for *Lactococcus lactis*: construction of an inducible thioredoxin gene fusion expression system.

23. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.

24. Sciarà, G., Blangy, S., Siponen, M., McGrath, S., van Sinderen, D., Tegoni, M., Cambillau, C., and Campanacci, V. (2008) A topological model of the baseplate of lactococcal phage Tuc2009. *J. Biol. Chem.* 283, 2716-2723.

25. Morath, S., Geyer, A., and Hartung, T. (2001) Structure-function relationship of cytokine induction by lipoteichoic acid from *Staphylococcus aureus*. *J. Exp. Med.* 193, 393-397.

26. Brauge, T., Sadovskaya, I., Faille, C., Benezech, T., Maes, E., Guerardel, Y., and Midlelet-Bourdin, G. (2016) Teichoic acid is the major polysaccharide present in the *Listeria monocytogenes* biofilm matrix. *FEMS Microbiol. Lett.* 10.1093/femsle/fnv229

27. Sadovskaya, I., Vinogradov, E., Li, J., and Jabbouri, S. (2004) Structural elucidation of the extracellular and cell-wall teichoic acids of *Staphylococcus epidermidis* RP62A, a reference biofilm-positive strain. *Carbohydr. Res.* 339, 1467-1473.

28. Vinogradov E, Sadovskaya I, Li J, and Jabbouri S. (2006) Structural elucidation of the extracellular and cell wall teichoic acids of *Staphylococcus aureus* MN8m, a biofilm forming strain. *Carbohydr. Res.* 341, 738–43.

29. Chapot-Chartier, M.-P., Vinogradov, E., Sadovskaya, I., Andre, G., Mistou, M.-Y., Trieu-Cuot, P., Furlan, S., Bidenko, E., Courtin, P., Péchoux, C., Hols, P., Dufrêne, Y.F., and Kulakauskas, S. (2010) Cell surface of *Lactococcus lactis* is covered by a protective polysaccharide pellicle. *J. Biol. Chem.* 285, 10464-10471.

30. Vinogradov, E., Sadovskaya, I., Cornelissen, A., and van Sinderen, D. (2015) Structural investigation of cell wall polysaccharides of *Lactobacillus delbrueckii* subsp. *bulgaricus* 17. *Carbohydr. Res.* 413, 93-99.

31. Chen, P. S., Toribara, T.Y., and Warner, H. (1956) Microdetermination of phosphorus. *Anal. Chem.* 28, 756-758.

32. Sadovskaya, I., Chaignon, P., Kogan, G., Chokr, A., Vinogradov, E., and Jabbouri, S. (2006) Carbohydrate-containing components of biofilms produced *in vitro* by some *Staphylococcus* strains related to orthopaedic prosthetic infections. *FEMS Immunol. Med. Microbiol.* 47, 75-82.

33. Marchler-Bauer, A., and Bryant, S.H. (2004) CD-search: protein domain annotations on the fly. *Nucleic Acids Res.* 32, W327-331.

34. Murphy, J., Bottacini, F, Mahony, J., Kelleher, P., Neve, H., Zomer, A., Nauta, A., and van Sinderen D. (2016) Comparative genomics and functional analysis of the 936 group of lactococcal *Siphoviridae* phages. Sci Rep. 10.1038/srep21345.

35. Szklarczyk,R. and Heringa,J. (2004) Tracking repeats using significance and transitivity. *Bioinformatics.* 20, I311–I317.

36. Shi, L., Liu, J.F., An, X.M., and Liang, D.C. (2008) Crystal structure of glycerolphosphodiesterase (GDPD) from *Thermoanaerobacter tengcongensis*, a metal ion-dependent enzyme: insight into the catalytic mechanism. *Proteins.* 72, 280-288.

37. Swoboda, J.G., Campbell, J., Meredith, T.C., and Walker, S. (2010) Wall teichoic acid function, biosynthesis, and inhibition. *ChemBiochem.* 11, 35-45.

38. Karamata, D., Pooley, H.M., and Monod, M. (1987) Expression of heterologous genes for wall teichoic acid in *Bacillus subtilis* 168. *Mol. Gen. Genet.* 207, 73-81.

39. Armstrong, J.J., Baddiley, J., and Buchanan, J.G. (1960) Structure of the ribitol teichoic acid from the walls of *Bacillus subtilis*. *Biochem. J.* 76, 610.
40. Larson, T.J., and van Loo-Bhattacharya, A.T. (1988) Purification and characterization of glpQ-encoded glycerophosphodiester phosphodiesterase from *Escherichia coli* K-12. *Arch. Biochem. Biophys.* **260**, 577-584.

41. Kusser, W. and Fiedler, F. (1982) Purification, Mr-value and subunit structure of a teichoic acid hydrolase from *Bacillus subtilis*. *FEBS Lett.* **149**, 67-70

42. Kusser, W. and Fiedler, F. (1983) Teichoicase from *Bacillus subtilis* Marburg *J. Bacteriol.* **155**, 302-310

43. Kusser, W. and Fiedler, F. (1984) A novel glycerophosphodiesterase from *Bacillus pumilus*. *FEBS Lett.* **166**, 301-306

44. Takeda, K., and Uetake, H. (1973) *In vitro* interaction between phage and lipopolysaccharide: a novel glycosidase associated with *Salmonella* phage ε15. *Virol.* **52**, 148-159

45. Lindberg, A.A., Wollin, R., Gemski, P., and Wohlieter, J.A. (1978) Interaction between bacteriophage Sf6 and *Shigella flexneri*. *J. Virol.* **27**, 38-44

46. Eriksson, U., Svenson, S.B., Lönngrren, J., and Lindberg, A.A. (1979) *Salmonella* phage glucanases: substrate specificity of the phage P22 endorhamnosidase. *J. Gen. Virol.* **43**, 503-511

47. Hughes, K.A., Sutherland, I.W., and Clark, J., and Jones, M.V. (1998) Bacteriophage and associated polysaccharide depolymerases – novel tools for study of bacterial biofilms. *J. Appl. Microbiol.* **85**, 583-590

48. Hanlon, G.W., Denyer, S.P., Olliff, C.J. and Ibrahim, L.J. (2001) Reduction in exopolysaccharide viscosity as an aid to bacteriophage penetration through *Pseudomonas aeruginosa* biofilms. *Appl. Environ. Microbiol.* **67**, 2746-2753.

49. Cornelissen, A., Ceyssens, P.J., T'Syen, J., Van Praet, H., Noben, J.P., Shaburova, O.V. Krylov, V.N., Volckaert, G., and Lavigne, R. (2011) The T7-related *Pseudomonas putita* phage φ15 displays virion-associated biofilm degradation properties. *PLoS One*. 1.0371/journal.pone.0018597

50. Hallenbeck, P.C., Virm, E.R., Yu, F., Bassler, B., and Troy, F.A. (1987) Purification and properties of a bacteriophage-induced endo-N-acetylneuramidase specific for poly-alpha-2,8-sialosyl carbohydrate units. *J. Biol. Chem.* **262**, 3553-3561

51. Stummeyer, K., Dickmanns, A., Mühlhennhof, M., Gerardy-Schahn, R., and Ficner, R. (2005) Crystal structure of the polysialic acid-degrading endosialidase of bacteriophage K1F. *Nat. Struct. Mol. Biol.* **12**, 90-96

52. Stummeyer, K., Schwarzer, D., Claus, H., Vogel, U., Gerardy-Schahn, R., and Mühlhennhof, M. (2006) Evolution of bacteriophages infecting encapsulated bacteria: lessons from *Escherichia coli* K1-specific phages. *Mol. Microbiol.* **60**, 1123-1135

53. Taylor, K. (1965) Enzymatic deacetylation of Vi-polysaccharide by Vi-phage. II. *Biochem. Biophys. Res. Commun.* **20**, 752-756

54. Taylor, K. (1966) Physical and chemical changes of Vi-polysaccharide due to Vi-phage II action. *Acta. Biochim. Polon.* **13**, 79-106

55. Caldentey, J., and Bamford, D.H. (1992) The lytic enzyme of the *Pseudomonas* phage f6. Purification and biochemical characterization. *Biochim. Biophys Acta.* **1159**, 44-50

56. Moak, M., and Molineux, I.J. (2000) Role of the Gp16 lytic transglycosylase motif in bacteriophage T7 virions at the initiation of infection. *Mol. Microbiol.* **37**, 345-355.

57. Rydman, P.S., and Bamford, D.H. (2000) Bacteriophage PRD1 DNA entry uses a viral membrane-associated transglycosylase activity. *Mol. Microbiol.* **37**, 356-363

58. Kenny, J.G., McGrath, S., Fitzgerald, G.F., and van Sinderen, D. (2004) Bacteriophage Tuc2009 encodes a tail-associated cell wall-degrading activity. *J. Bacteriol.* **186**, 3480-3491

59. Stockdale, S.R., Mahony, J., Courtin, P., Chapot-Chartier, M.P., van Pijkeren, J.P., Britton, R.A., Neve, H., Heller, K.J., Aideh, B., Vogensen, F.K., and van Sinderen, D. (2013) The lactococcal phages Tuc2009 and TP901-1 incorporate two alternate forms of their tail fiber into their virions for infection specialization. *J. Biol. Chem.* **288**, 5581-5590

60. Coyette, J., and Ghysen, J.-M. (1968) Structure of the cell wall *Staphylococcus aureus*, strain Copenhagen. IX. Teichoic acid and phage adsorption. *Biochemistry.* **7**, 2385-2389

61. Chatterjee, A. N. (1969) Use of bacteriophage-resistant mutants to study the nature of the bacteriophage receptor site of *Staphylococcus aureus*. *J. Bacteriol.* **98**, 519-527
62. Archibald, A. R. (1976) Cell wall assembly in Bacillus subtilis. Development of bacteriophage binding properties as a result of the pulsed incorporation of teichoic acids. J. Bacteriol. 27, 956-990
63. Givan, A. L., Glassey, K., Green, R. 5., Lang, W. K., Anderson, A. I. and Archibald, A. R. (1982) Relation between wall teichoic acid content of Bacillus subtilis and efficiency of adsorption of bacteriophages SP50 and 425. Arch. Microbiol. 33, 318-322
64. Baptista, C., Santos, M.A., and Săo-José, C. (2008) Phage SPP1 Reversible adsorption to Bacillus subtilis cell wall teichoic acids accelerates virus recognition of membrane receptor YueB. J. Bacteriol. 190, 4989-4996
65. Wendlinger G, Loessner MJ, Scherer S. (1996) Bacteriophage receptors on Listeria monocytogenes cells are the N-acetylglucosamine and rhamnose substituents of teichoic acids or the peptidoglycan itself. Microbiology. 142, 985-992
66. Munsch-Alatossava, P., and Alatossava, T. (2013) The extracellular phage-host interactions involved in the bacteriophage LL-H infection of Lactobacillus delbrueckii ssp. lactis ATCC 15808. Front. Microbiol. 4, 1-5
67. Rousseau, G.M., and Moineau, S. (2009) Evolution of Lactococcus lactis phages within a cheese factory. Appl. Environ. Microbiol. 75, 5336-5344
68. Castro-Nallar, E., Chen, H., Gladman, S., Moore, S.C., Seemann, T., Powell, I.B., Hillier, A., Crandall, K.A., and Chandry, P.S. (2012) Population genomics and phylogeography of an Australian dairy factory derived lytic bacteriophage. Genome Biol. Evol. 4, 382-393
69. Kornspan, J.D., and Rottem, S. (2012) Phospholipase A and glycerophosphodiesterase activities in the cell membrane of Mycoplasma hyorhinis. FEMS Microbiol. Lett. 332, 34-39
70. Simocková, M., Holic, R., Tahotná, D., Patton-Vogt, J., and Griac P. (2008) Yeast Pgc1p (YPL206c) controls the amount of phosphatidylglycerol via a phospholipase C-type degradation mechanism. J. Biol. Chem. 283, 17107-17115
71. Sundaralingam, M., and Jensen, LH. (1965) Crystal and molecular structure of a phospholipid component: L-alpha-glycerophosphorylcholine cadmium chloride trihydrate. Science. 150, 1035-1036
FOOTNOTES
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FIGURE LEGENDS

FIGURE 1. In silico domain analysis of GDPD_{Ld17} and GDPD_{340}. (A) Modular structure of GDPD_{Ld17} and GDPD_{340} based on NCBI Conserved Domain Detection analysis (33). The predicted catalytic domains (glycerophosphodiester phosphodiesterase (GDPD; cd08556, 5.1E^{-38}) and GDPD_like_2 (cd08582, 7.40E^{-15})) are indicated as dark grey boxes. Sequence similarity (GDPD_{Ld17}, 9.86E^{-27}; GDPD_{340}, 2.65E^{-09}) to the GDPD, UgpQ, of *Escherichia coli* is depicted. The predicted carbohydrate-binding (CB) domain (CBM_4_9; 9.76E^{-03}) of GDPD_{Ld17} and the internal repeat structure of the putative CB-domain of GDPD_{340}, as predicted by the Trust server (35), are indicated by black boxes. The extreme N-terminal domain of GDPD_{340}, probably involved in physical association with the virion, contains for GDPD_{340} a DUF2479 domain (light gray box; 9.76E^{-03}) which is typically found in the neck-passage structure of lactococcal 936-type phages. The double arrows delineate the modules designated as “catalytic” and “catalytic + carbohydrate-binding” as used in further experiments. (B) ClustalW comparison of the UgpQ-like GDPD-domains (amino acids in light grey were excluded from the NCBI CDD delineation). The putative catalytic histidine residues are marked in black, while the glutamic acid and aspartic acid residues involved in binding of the divalent cations are marked in dark grey. Identical residues are marked by an asterisk, while residues marked with a point and colon display chemical and physical similarity which is higher for the latter. Gaps, indicated by horizontal lines, were introduced into the sequences to maximize the alignment.

FIGURE 2. Transmission electron micrographs of immunogold-labelled GDPD protein as part of the baseplate of *Lb. delbreuckii* subp. *bulgaricus* phage 17 (A) and as part of the neck passage structure of *Lc. lactis* ssp. *lactis* phage 340 (B). Phages were incubated with polyclonal anti-GDPD rabbit antibodies raised against the GDPD specific for that phage and labelled with anti-rabbit secondary mouse antibodies conjugated to 5-nm gold particles.

FIGURE 3. GDPD_{Ld17} and GDPD_{340} are trimeric proteins. (A) SEC-MALS-RI chromatogram of the GDPD_{Ld17} (111.9 kDa theoretical molecular weight, left) and GDPD_{340} (110.7 kDa theoretical molecular weight, right). The molar mass (left axis, solid line) and the UV_{280nm} absorbance (right axis, dotted line) are plotted as function of the column elution volume. (B) Protein bands of GDPD_{Ld17} and GDPD_{340} on a 7 % (w/v) SDS-PAGE gel with (95 °C, 5 min) and without (RT, 5 min) boiling prior to electrophoresis.

FIGURE 4. NMR analysis of the GDPD_{Ld17}-treated Gro3P-substituted linear D-galactan (sPS2) of Ldb17. Chemical structure (30) and part of the HSQC spectrum of the Ldb17 sPS2 (green-black) and GDPD_{Ld17}-treated Ldb17 sPS2 (red-violet). Note the absence of Gro and A’ signals in the GDPD_{Ld17}-treated sPS2 spectrum.
FIGURE 1.
FIGURE 2.
FIGURE 3.
FIGURE 4.

\[-2\text{-b-Gal}^f\text{-3-a-Gal}\rho\text{-2-b-Gal}^f\text{-6-b-Gal}^f\text{-3-b-Gal}\rho\text{-}6\]

\((\text{Gro}3\text{P})\)
TABLE 1. Enzymatic activity of GDPD$_{Ld17}$ on purified carbohydrates. GDPD-activity expressed as \( [\Delta \text{340nm}/(\mu \text{mol GDPD} \times \text{min})] \) was evaluated using the enzyme-coupled spectrophotometric assay on 1.5 g/l purified carbohydrates. Average and standard deviation of three repeats are given. HPAEC-PAD chromatograms of purified carbohydrates (2.5 g/l) exposed to GDPD$_{Ld17}$ at 30°C for 2 h detected only Gro3P as end product of GDPD$_{Ld17}$-activity.

|                          | Reference for chemical structure | GDPD-activity \([\Delta \text{340nm}/(\mu \text{mol} \times \text{min})]\) | HPAEC-PAD |
|--------------------------|---------------------------------|-------------------------------------------------|-----------|
| **Cell wall teichoic acid** |                                 |                                                 |           |
| *Staphylococcus aureus* NM8m | (28)                            | 137.33 ± 1.33                                   | Gro3P     |
| *Staphylococcus epidermidis* 1457 | -                               | 172.44 ± 5.05                                   | Gro3P     |
| *S. epidermidis* RP62A       | (27)                            | 11.11 ± 0.77                                    | Gro3P     |
| *Lb. delbrueckii* ssp. *bulgaricus* 17 | (30)                          | 31.56 ± 0.77                                    | Gro3P     |
| *Bacillus subtilis* 168      | (37)                            | 56.67 ± 3.45                                    | Gro3P     |
| *Bacillus subtilis* W23      | (39)                            | 3.20 ± 0.79                                     | -         |
| **Extracellular teichoic acid** |                                 |                                                 |           |
| *Listeria monocytogenes* ScottA | -                              | 5.33 ± 1.33                                     | -         |
| **Lipoteichoic acid**        |                                 |                                                 |           |
| *S. aureus* (Sigma-aldrich)  | -                               | 5.33 ± 1.33                                     | -         |
| *B. subtilis* (Sigma-aldrich) | -                              | 6.66 ± 1.33                                     | -         |
| *Lc. lactis* ssp. *cremonis* MG1363 | -                           | 5.33 ± 2.31                                     | -         |
| **Cell wall-associated polysaccharide** |                         |                                                 |           |
| *Lc. lactis* ssp. *cremonis* MG1363 – pellicle | (29)                      | 2.22 ± 2.04                                     | -         |
| *Lb. delbrueckii* ssp. *bulgaricus* 17 - hydrophobic PS1 | (30)                  | 3.33 ± 1.92                                     | -         |
| *Lb. delbrueckii* ssp. *bulgaricus* 17 - galactan with Gro3P-decoration | (30)                  | 873.33 ± 13.33                                  | Gro3P     |
| **Glycerophosphorylcholine** | (71)                            | 2753.33 ± 34.64                                 | Gro3P     |
**TABLE 2.** Influence of 20 mM divalent cations on phage Ld17 characteristics. (A) The native GDPD-activity present in the Ld17 virion was assayed in 50 mM MOPS pH 7.5, 300 mM NaCl on 2.5 mM GPC substrate after incubation at 30 °C for 2 h with the enzyme-coupled spectrophotometric assay. 100 % activity is set equal to the condition without additional divalent cations. (B) Adsorption of phage Ld17 on its host Ldb17. The adsorption is expressed as the ratio of adsorbed phage after 10 min to the number of free phage at time point zero. (C) The relative efficiency of plating (EOP) of phage Ld17 on its host Ldb17 compared to the optimal condition containing 20 mM CaCl$_2$. Relative EOP were not determined (ND) for Cd$^{2+}$ and Zn$^{2+}$ due to the absence of bacterial growth of the Ldb17 host under these conditions. For each condition, the average and standard deviation of three independent experiments is given.

|         | Relative activity (%) | Adsorption (%) | Relative EOP |
|---------|-----------------------|----------------|-------------|
| None    | 100.0 ± 1.0           | 75.7 ± 3.8     | 0.00 ± 0.00 |
| Ca$^{2+}$ | 127.2 ± 2.0          | 91.0 ± 0.2     | 1.00 ± 0.16 |
| Mg$^{2+}$ | 32.8 ± 1.1           | 71.1 ± 4.9     | 0.57 ± 0.04 |
| Mn$^{2+}$ | 0.2 ± 0.2            | 64.6 ± 1.8     | 0.40 ± 0.03 |
| Co$^{2+}$ | 0.0 ± 0.9            | 44.8 ± 3.6     | 0.00 ± 0.00 |
| Ni$^{2+}$ | 0.0 ± 0.2            | 64.7 ± 6.2     | 0.00 ± 0.00 |
| Cd$^{2+}$ | 0.0 ± 0.2            | 48.6 ± 6.1     | ND          |
| Zn$^{2+}$ | 0.0 ± 0.2            | 75.7 ± 3.8     | ND          |
TABLE 3. Enzymatic activity of GDPD<sub>Ld17</sub> on a diverse set of CWaC-extracts. GDPD-activity expressed as $[\Delta_{340\text{nm}}/(\mu\text{mol GDPD} \times \text{min})]$ was evaluated using the enzyme-coupled spectrophotometric assay on 1.5 g/l CWaC-extracts. Average and standard deviation of three repeats are given.

| Lb. delbrueckii subp. bulgaricus strains | GDPD-activity $[\Delta_{340\text{nm}}/(\mu\text{mol} \times \text{min})]$ |
|-----------------------------------------|-------------------------------------------------|
| Ldb9                                   | 697.78 ± 7.70                                   |
| Ldb10                                  | 1402.22 ± 13.88                                 |
| Ldb2                                   | 480.00 ± 6.67                                   |
| Ldb14                                  | 1522.22 ± 15.40                                 |
| Ldb41                                  | 873.33 ± 6.67                                   |
| Ldb40                                  | 337.78 ± 16.78                                  |
| Ldb17                                  | 962.22 ± 10.18                                  |
| Ldb36                                  | 508.89 ± 34.21                                  |
| Ldb22                                  | 328.89 ± 25.24                                  |
| Ldb34                                  | 1024.44 ± 20.37                                 |
| Ldb35                                  | 860.00 ± 61.10                                  |
| Ldb66                                  | 504.44 ± 27.76                                  |