Title: The peptidoglycan hydrolase of the *Staphylococcus aureus* bacteriophage Φ11 plays a structural role in the viral particle.

Running title: The structural role of Φ11 peptidoglycan hydrolase

Authors: Lorena Rodríguez-Rubio¹, Nuria Quiles-Puchalt², Beatriz Martínez¹, Ana Rodríguez¹, José R. Penadés² and Pilar García¹#

Addresses: ¹DairySafe Group. Department of Technology and Biotechnology of Dairy Products. Instituto de Productos Lácteos de Asturias (IPLA-CSIC), 33300 Villaviciosa, Asturias, Spain.
²Instituto de Biomedicina de Valencia (IBV-CSIC), 46010, Valencia, Spain.

#Corresponding author: Dr. Pilar García

IPLA-CSIC, Paseo Río Linares, 33300-Villaviciosa, Asturias, Spain.

e-mail: pgarcia@ipla.csic.es

Phone: +34 985 89 21 31

Fax: +34 985 89 22 33
Abstract

The role of virion-associated peptidoglycan hydrolases (VAPGH) in the phage infection cycle is not clear. Gp49, the VAPGH from Staphylococcus aureus phage Φ11 is not essential for phage growth but stabilizes the viral particles. Φ11Δ49 phages showed reduced burst size and delayed host lysis. Complementation of gp49 with HydH5 from bacteriophage vB_SauS-phiIPLA88 restored the wild type phenotype.
Bacteriophages are provided with peptidoglycan hydrolases (VAPGH) able to disrupt the peptidoglycan (PG) layer to allow the delivery of phage DNA into the cell (1, 2). Cell wall degradation by these proteins is restricted to local hydrolysis of the PG layer once the virion is adsorbed to the bacterial surface. However, these proteins are also responsible for the “lysis from without”, a bacterial lysis induced by high-multiplicity virion adsorption that occurs without phage production (3). Although the PG hydrolase activity of these proteins has been demonstrated, it is not clear their function during phage lytic cycle. PRD1 and T7 mutant phages without VAPGH activity were able to infect host cells although the process was significantly delayed (4, 5). Likewise, phage infection was carried out by T4 mutant phages harbouring a reduced lysozyme activity (6). On the contrary, antibody neutralization studies against a tail-associated protein of *L. lactis* bacteriophage Tuc2009 resulted in the host lysis inhibition by more than 100-fold (7). Moreover, it has been reported that the tape measure protein of mycobacteriophage TM4 that harbours a PG hydrolase motif, enables phage infection in *Mycobacterium smegmatis* during the stationary phase (8, 9).

In a similar way, the transglycosylase activity of bacteriophage T7 gp16 is shown to be beneficial during infection of *Escherichia coli* cells grown to high cell density and also important for phage growth at temperatures below 20°C (4). Recently, it has been showed that Tuc2009 and TP901-1 virions may contain either full-length or truncated VAPGHs, in order to infect bacteria with different levels of cell wall cross-linkage (10).

Sequence analysis of phage Φ11 showed a putative VAPGH (gp49) in the late region (11), which muralytic activity was previously detected in zymograms against *S. aureus* cells using 10^10 phages (1). The complete genome sequence and zymogram analysis of the *S. aureus* bacteriophage vB_SauS-phiPLA88 revealed the presence of HydH5, a VAPGH able to lyse viable *S. aureus* cells (12). HydH5 (634 amino acids,
Acc. Number ACJ64586) showed 91% similarity with gp49 (632 amino acids, Acc. 
Number NP_803302.1) and the same catalytic domains (CHAP and LYZ2) (12). In this 
work, the effects of the lack of gp49, and the subsequent complementation with HydH5, 
on both Φ11 viability and structure were determined.

**Gp49 is not essential for phage growth.** The strain *S. aureus* RN451 (φ11 
lysogen) was used to obtain mutant phages (φ11Δ49) without gp49 (13) by allelic 
exchange using derivatives of plasmid pMAD (28) carrying the desired mutations (14).  
The oligonucleotides used were: orf49phi11-1mB (5’- 
CGCGGATCCCTTAAGTGTTAGCGAGAAGGG- 3’); orf49phi11-2c (5’-
TGCTCCCATTCCAGAACAGG- 3’); orf49phi11-3m (5’-
CTGGTTCGTGAATGGGAGCAGCTACTGCTATAGAGTGGTGC- 3’); and 
orf49phi11-4cE (5’-CCGGAAATCTTTTTGCTCCAGGTGTCCCGG- 3’). Lysates 
containing Φ11 and Φ11Δ49 mutant particles were prepared by mitomycin C (2 µg/ml) 
induction of lysogenic strains *S. aureus* RN451 and *S. aureus* JP4049 (RN450 lysogenic 
for Φ11Δ49), respectively, grown in TSB broth (Tryptic Soy Broth, DIFCO, Franklin 
Lakes, NJ) (14). For enumeration of phages by the double layer technique (15) dilutions 
were made in Phage Buffer (1 mM NaCl, 0.05 mM Tris-HCl, 1 mM MgSO₄, 4 mM 
CaCl₂, pH 7.8), and *S. aureus* RN4220 grown in Nutrient Broth (NB) (Oxoid, 
Basingstoke, UK) at 37°C used as indicator (16). Diameter of Φ11 lysis plaques was 1.1 
± 0.2 mm, while Φ11Δ49 lysis plaques were smaller than 1 mm (Fig. 1A). The presence 
of Φ11Δ49 lysis plaques suggests that gp49 is not essential for life cycle. However, the 
phage titre of Φ11Δ49 suspensions (2.18 × 10⁸ PFU/ml) was 10-fold lower than that of 
Φ11 suspensions (2.2 × 10⁹ PFU/ml). This result is consistent with previous studies and 
could indicate that an increase of the PG turnover occurs when cells are growing 
exponentially (17), allowing the tail structure to pass through.
The analysis of the single-step growth curve (18) of Φ11Δ49 propagated on *S. aureus* RN4220 showed a similar latent period (10 min) than Φ11 (Fig. 1B). However, the burst size of Φ11Δ49 ranged between 9 and 11 PFU per infected cell in comparison with 15-23 PFU per infected cell detected in Φ11. Moreover, our results are in total concordance with previous observations in relation to the plaque size, which seems to be directly proportional to the burst size and diffusion of phages in the culture medium, and inversely proportional to the latent period and the adsorption rate, each factor contributing differently (19, 20).

**Φ11Δ49 particles are unstable and lose their DNA.** Bacteriophages Φ11 and Φ11Δ49 were purified by a CsCl$_2$ continuous density gradient (21) and negatively stained with 2% uranyl acetate. Transmission electron microscopy observations (JEOL 12.000 EXII; JEDL USA Inc, Peabody, MA, USA) showed virions with identical features: isometric capsid (57.5 ± 3.4 nm), long and narrow non-contractile tail (177.7 ± 7.3 nm), and a baseplate with short tail fibers (Fig. 2). However, about 93.3% of Φ11Δ49 particles had empty heads, indicating that they had lost their DNA despite the fact that tails were not moved apart (Fig. 2B). As far as we know, this is the first time that an additional structural role is proposed for a VAPGH. This result might not be surprising since it has been previously reported that T4 gp5 lysozyme is an essential structural component of T4 baseplate. In fact, the proper tail assembly requires a sequential association of lysozyme subunits (22). However, the lack of gp49 did not affect the proper assembly of the Φ11Δ49 virion particle, as judged by the purification of these particles in a CsCl$_2$ gradient. Therefore, we can speculate that gp49 might be located in the baseplate, where it might play a role in keeping the DNA inside the head, in a similar way to some mechanisms previously described (23).
Lack of gp49 is not involved in phage adsorption but delays host lysis by Φ11. Kinetics of phage binding to *S. aureus* RN4220 cells were measured (24) using a MOI of 0.01. Adsorption of phage Φ11 proceeded up to 85% in 15 min and similar values were observed for Φ11Δ49 (data not shown). Preliminary challenge trials were performed using *S. aureus* RN4220 exponential cultures (OD₆₀₀nm = 0.1) (10⁷ CFU/ml) infected at MOI 0.01 and incubated at 37°C for 8 h, and at room temperature for 24 h. Within the first 100 min, the optical density was similar for both Φ11 and Φ11Δ49 infected cultures. Afterwards, the OD of both RN4220 infected cultures was reduced dramatically, but a delay of 15 min was observed in Φ11Δ49 infected cultures compared with those infected by Φ11 infected (Fig. 3A). The difference between the lytic cycle of both phages was more pronounced when infection took place at lower temperatures since a delay of 3.5 h was observed in Φ11Δ49 infected cultures (Fig. 3B). Moreover, a complete lysis did not occurred since OD₆₀₀nm was only reduced from 0.5 to 0.35 in 12 h after the onset of lysis. Delay in the culture lysis could be explained by the lower production of viable particles in Φ11Δ49 phages. In addition, the delay at lower temperature might be indicative of some difficulties in the infection process under these conditions. In this regard, we cannot discard the involvement of gp49 in the ability of Φ11 to pass through (overcome) the peptidoglycan layer. Murein layer is known to be more highly cross-linked under some growing conditions (25), and the peptidoglycan hydrolase gp49 might be necessary for phage Φ11 to infect under suboptimal conditions.

**Lack of gp49 in Φ11Δ49 phages can be complemented by phiIPLA88**

**HydH5.** The sequence homology between HydH5 from phiIPLA88 and gp49 from Φ11 allowed us to complement the Φ11 mutation with HydH5. The HydH5 or gp49 encoding genes were amplified using the oligonucleotides orf49Φ11-9mB (5’-CGCGGATCCAGTTAAGAGTCAGTGCTTCG-3’) and orf49Φ11-10cE (5’-CGCGGATCCAGTTAAGAGTCAGTGCTTCG-3’)
CCGGAATTCATTTTGTACATTACACACCTC-3′) and either phiIPLA88 DNA (26) or Φ11 DNA were used as template. PCR amplifications were carried out using the PFU polymerase (Promega, Madison, WI, USA), and then PCR fragments were purified using the GenElute PCR clean-up kit (Sigma Missouri, USA). The resulting PCR products (1984 bp or 1987 bp, respectively) were cleaved with BamHI/EcoRI restriction enzymes (Takara, Otsu, Shiga, Japan) and cloned into plasmid pCN51 (26) to generate pCN51-orf58phiIPLA88 and pCN51-orf49Φ11. E. coli DH5α was used to select recombinant plasmids in LB plates (Luria-Bertani broth, Pronadisa, Madrid, Spain) supplemented with ampicillin (100 µg/ml). The resulting plasmids were electroporated into S. aureus RN4220 and phage Φ11 was used to transduce them into the appropriate strains. Cadmium inducible expression plasmids pCN51-orf49Φ11 and pCN51-orf58phiIPLA88 were transduced into S. aureus JP4049 (lysogenic for Φ11Δ49) to obtain the derivative strains S. aureus JP8671 [JP4049 (pCN51-orf49Φ11)] and S. aureus JP8758 [JP4049 (pCN51-orf58phiIPLA88)], respectively. Overnight cultures were inoculated at 1% (v/v) in fresh TSB containing 5 µM CdCl₂ to ensure the presence of the proteins in the host cytoplasm when phages particles were ready to be assembled, grown to OD₆₀₀nm = 0.3 and induced by adding mitomycin C (2 µg/ml). S. aureus RN4220 (pCN51-orf49Φ11) and S. aureus RN4220 (pCN51-orf58phiIPLA88) were also grown in the presence of 5 µM CdCl₂ and used to obtain complemented phages suspensions. After complementation, the wild type lysis plaque size (1.1 ± 0.2 mm) was recovered in both complementation assays. When Φ11Δ49 mutant phages were complemented with HydH5 protein, plaques showed an average diameter of 1.2 ± 0.2 mm, whereas complementation with gp49 resulted in plaques of 1.1 ± 0.2 mm. Regarding the phage titre, suspensions containing mutant phages complemented with HydH5 protein had a titre of 1.34 × 10⁹ PFU/ml, whereas 1.2 × 10⁹ PFU/ml was the titre
of suspensions containing mutant phages complemented with gp49 protein. Of note, all of the phage particles kept the DNA inside the heads regardless of Φ11Δ49 was complemented either with gp49 or HydH5 (Fig. 2C). This observation is in concordance with the involvement of similar structural phage proteins in the DNA transfer into the host (23). Complementation studies clearly demonstrated the ability of HydH5 to counteract the lack of gp49 in Φ11 particles, suggesting a similar role of these proteins in both phages. Most of the information regarding the function and structure of proteins involved in host attachment and cell wall penetration comes from phages with contractile injection systems like phage T4, which is provided with a special spike-shaped protein complex, the “cell-puncturing device”, used to pierce the outer cell membrane in the process of tail sheath contraction (27). In phages with non-contractile tail, no other baseplate proteins involved in passing through the cell wall and bacterial membrane have been reported. The function of gp49 and HydH5 is still unknown, but their presence at the tail morphogenetic module might suggest their implication in the adsorption and infection process. Overall, our findings show that Φ11 gp49 is not essential for the phage infection cycle, at least under normal laboratory conditions. However, a structural role in the virions might be deduced from the instability of particles lacking this protein. Complementation assays of gp49 with its homologue HydH5 were successful and support a similar role for both virion-associated proteins.

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Figures

**Figure 1.** Main differences between Φ11 and Φ11Δ49 life cycles. A) Morphology of Φ11 and Φ11Δ49 lysis plaques. B) Single-step growth curve of phages Φ11 (■) and Φ11Δ49 (○) on exponential cultures of *S. aureus* RN4220. Error bars show the means ± standard deviations of three independent assays.

**Figure 2.** Electron micrographs of phages Φ11 (A), Φ11Δ49 (B), Φ11Δ49 complemented with HydH5 protein (C). Phages were negatively stained with 2% uranyl acetate. The arrow indicates a complete Φ11Δ49 particle containing DNA.

**Figure 3.** Lysis of *S. aureus* RN4220 by Φ11 (■) and Φ11Δ49 (●) phages at A) 37°C and B) room temperature. *S. aureus* RN4220 without phages was used as control (▲). Error bars show the means ± standard deviations of three independent assays.
Φ11Δ49 complemented with HydH5