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Identification of EPS-degrading activity within the tail spikes of the novel *Pseudomonas putida* phage AF

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ABSTRACT

We report the study of phage AF, the first member of the canonical lambdoid phage group infecting *Pseudomonas putida*. Its 42.6 kb genome is related to the "epsilon15-like viruses" and the "BPP-1-like viruses", a clade of bacteriophages shaped by extensive horizontal gene transfer. The AF virions display exopolysaccharide (EPS)-degrading activity, which originates from the action of the C-terminal domain of the tail spike (Gp19). This protein shows high similarity to the tail spike of the T7-like *P. putida*-infecting phage φ15. These unrelated phages have an identical host spectrum and EPS degradation characteristics, designating the C-terminal part of Gp19 as sole determinant for these functions. While intact AF particles have biofilm-degrading properties, Gp19 and non-infectious AF particles do not, emphasizing the role of phage amplification in biofilm degradation.

P. putida is a metabolic versatile soil bacterium which is applied in bioremediation of contaminated soils, biocatalysis of specific chemicals, biopesticides and as plant growth promoting agents (Dejonghe et al., 2001; Lehrbach et al., 1984; Timmis et al., 1994). Human infections caused by *P. putida* have been reported in immunocompromised patients (Bouallègue et al., 2004; Ladhani and Bhutta, 1998) and patients with invasive medical devices (Martino et al., 1996). The ability to form a biofilm, a protective cell-enclosed environment, is a key feature in the pathogenicity of *P. putida* (von Eiff et al., 2005).

In the search for novel anti-biofilm agents, there is a rising interest in the biofilm-degrading properties of phages. Adams and Park already reported in 1956 the phages which display this enzymatic tail-associated activity to enable them to reach the bacterial cell surface receptors (Adams and Park, 1956). For a long time, research on these phage enzymes was focused on the capsulated neuroinvasive *Escherichia coli* K1 strains (Bull et al., 2010; Scholl et al., 2005). Very recently, novel phages infecting *E. coli* and *Acinetobacter baumannii* strains were isolated and shown to degrade bacterial biofilms (Chibeu et al., 2012; Yele et al., 2012). Likewise, the isolation and preliminary characterization of biofilm-degrading phages targeting *Pseudomonas* species was reported (Glonti et al., 2010; Shaburova et al., 2009). Among these, the T7-related phage φ15 was found to contain EPS-degrading tail spike proteins (Cornelissen et al., 2011; Shaburova et al., 2009). Here, we describe the genomic and microbiological characterization of *P. putida* phage AF originally isolated from a soil sample in Chicago (Illinois, USA) in 1977 by Shaburova et al. (2009).

Phage characteristics and halo formation

Based on structural characteristics, AF can be classified within the *Podoviridae* family of short-tailed phages consisting of isometric heads and short noncontractile tails with tail spikes lying at each side (59.9 nm head; 12 × 8 nm tails) (Supplementary Fig. 1A). Phage AF is stable in a relatively broad pH range with more than 66.0% infective phage particles after 24 h incubation at pH 5–11 and about 11.5% at the extreme boundaries of pH 4 and 12 (Supplementary Fig. 1B).Within the first minute of infection, 97.3% of administered AF virions adsorbed irreversibly to *P. putida* host PpG1 (k_a=2.34E−7) (Supplementary Fig. 1C). PpG1 infected host cells are lysed 50 min post infection, releasing on average 53 new phage particles (Supplementary Fig. 1D).

Host range analysis (Gill et al., 2003) on a set of 53 *P. putida* strains (described in Cornelissen et al. (2011)) identified three *P. putida* strains – PpG1, PpN and PpN3 – on which AF forms small clear plaques, while small turbid plaques are formed on four rice rhizosphere strains (RD6PR1, RD5PR2, RD8PR2 and RD8PR3). Independent of the type of plaques formed, AF zones of infection

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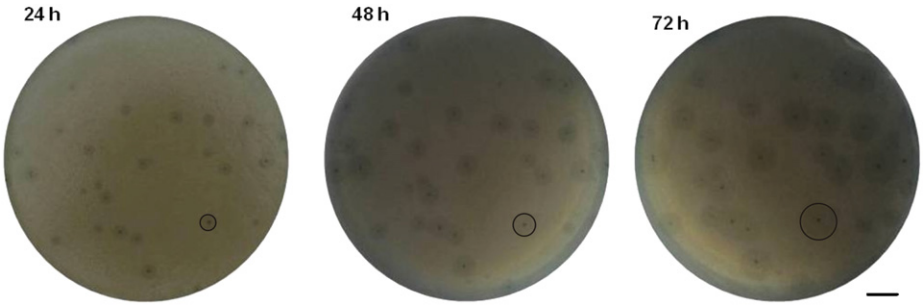

Fig. 1. Plaques of *P. putida* phage AF on the host PpG1. With increasing incubation at 30 °C, the diameter of the halo zone, surrounding the plaque with constant diameter, increases every 24 h. Scale bar represents 1 cm.

are always surrounded by an opaque-looking halo zone which increases in diameter upon overnight incubation at 30 °C (Fig. 1). Interestingly, these seven AF-susceptible strains match perfectly with the host range of the T7-related phage φ15 (Cornelissen et al., 2011). To investigate these halo zones, 10^8 pfu of phage AF was dropped on a bacterial lawn of PpG1. In contrast to the central zone of infection, which retained its diameter, the halo zone expanded up to 3 mm in diameter every 24 h. On equally large surface areas, only about a twenty fold less phage was identified in the halo zone ($1.65 \pm 1.46E+6$ pfu/cm^2) compared to the lysis zone ($3.37 \pm 2.30E+7$ pfu/cm^2), while no phages were observed outside the halo zone. Conversely, no viable bacterial cells were observed within the lysis zone (except for the few resistant colonies), while the halo zone ($5.10 \pm 2.25E+7$ CFU/cm^2) and the outside bacterial zone ($4.58 \pm 2.32E+7$ CFU/cm^2) contain almost equal bacterial counts. So, both infective phage particles and viable bacterial cells are present in the halo zone of phage AF. This is consistent with the observations for *P. putida* phage φ15 (Cornelissen et al., 2011) and suggest an inability or decreased ability of phage AF to replicate on bacterial cells in the stationary phase of growth. The halo zone hints at the presence of a virion-associated tail spike which degrades the bacterial EPS layer. Phage diffusion out of the lysis zone over time further explains the increasing diameter of the halo zone.

Genome and proteome analysis of phage AF

The AF genome (GenBank accession number JX676771) encompasses 42,689 bp encoding 65 gene products, and is tightly organized with a predicted 3.5% noncoding sequence and 35 overlapping ORFs (Fig. 2, Supplementary Table 1). The overall GC-content of 58.44% is only slightly lower than that of *P. putida* genomes (61% G+C). However, six early genes display an atypical low G+C average ($\leq$51%) (Supplementary Table 1). These genes were probably acquired recently through horizontal gene transfer from an A+T rich organism and do not display any sequence similarity, except for the HNH endonuclease Gp36 which is similar to *Lactobacillus* phage gene products. No tRNA genes were predicted.

The genome is organized into two major clusters (left and right arms) that differ according to the direction of transcription. The early/middle region consists of a rightward expression unit of seventeen ORFs predominately involved in DNA replication, while the leftward recombination expression unit consists of 23 ORFs. Apart from limited similarity to the *P. putida* GB-1 prophage (4 gene products), these genes are similar to genes of (pro)phages infecting all three families of the *Caudovirales*. In this region, AF encodes protein (domains) typical for members of the lambdoid phage group. These include the restriction alleviation protein Lar (Gp28), two proteins involved in recombination (ERF family protein (Gp39) and NinB (Gp37)), domains of the replication protein O and the CI repressor homologous to the N-terminal parts of Gp49 and Gp51 (Fig. 2; Supplementary Table 1).

The late region involved in virion morphogenesis and host cell lysis is encoded by the rightward unit of expression, comprising 25 putative genes (gene *1* to *25*). It displays high similarity to the "epsilon15-like viruses" (the *Salmonella enterica* serovar Anatum-specific phage ε15 (Kropinski et al., 2007) and the *E. coli* O157:H7-specific phage φV10 (Perry et al., 2009)), the "BPP-1-like viruses" (the *Burkholderia cepacia*-specific phage BcepC6B and the *Bordetella bronchiseptica*-specific BPP-1 related phages (Liu et al., 2004)) and the putative *P. putida* GB-1 prophage. Arrangement of these homologous genes is identical, but subjected to deletions, insertions and replacements (Supplementary Table 1). Using ESI-MS/MS analysis of CsCl purified phage particles (Ceyssens et al., 2008), 21 AF gene products were identified as being part of the structural particle (diamonds, Fig. 2). Twelve of these represent predicted structural gene products. Interestingly, nine proteins encoded by genes dispersed throughout the early and middle regions of the AF genome were picked up by MS analysis. As we used a phage preparation which was purified by CsCl purification, the possibility that these proteins are impurities is small. However, the role of these proteins and the reason of incorporation into the phage particle is unknown.

The AF tail spike protein displays EPS-degrading activity

Gene 19 encodes the tail spike protein of phage AF. This tail spike contains an N-terminal domain which is widely conserved within the tail spike homologs of all members of the "epsilon15-like viruses" and the "BPP-1-like viruses" (Supplementary Table 1) and the "T7-like viruses" (Pfam; AA 1–170; phage T7_tail; PF03906; 7.6E−23). It serves to connect the C-terminal catalytic module interacting with the host cell receptor and the phage tail (Garcia-Doval and van Raaij, 2012; Steven et al., 1988). Moreover, similarity to the T7 N-terminus extends further into the C-terminal domain of the tail spike protein Gp17 of the T7-like phage φ15 resulting in an overall protein similarity (BLASTP; *E*-value 0.0; 48% identity) (Supplementary Fig. 2). While the C-terminal moiety of both "epsilon15-like viruses" shows endorhamnosidase activity degrading the O-antigen of the bacterial outer membrane (Kanegasaki and Wright, 1973; Takeda and Uetake, 1973), the C-terminal domain of φ15-Gp17 is probably involved in EPS degradation (Cornelissen et al., 2011). Based on this observation it is reasonable to assume that the tail spike protein of phage AF also performs this EPS-degrading activity.

To analyze the catalytic activity of AF-Gp19, we cloned the C-terminal gene segment (*gp19NΔ137*) (5′-ATGGATTACTTCGA-CGCTCTC-3′ and 5′-CTACCCGAGAAGATCAACCA-3′; Eurogentec, Seraing, Belgium) into the pEXP5-NT/TOPO® vector (Invitrogen Corporation), with a 6 × His-tag to facilitate protein purification. Expression occurred at 16 °C overnight in *E. coli* BL21(DE3)pLysS