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MICROBIAL GENETICS

TL, the New Bacteriophage of *Pseudomonas aeruginosa* and Its Application for the Search of Halo-Producing Bacteriophages

E. A. Pleteneva[a], M. V.Bourkaltseva[a], O. V. Shaburova[a], S. V. Krylov[a], E. V. Pechnikova[b], O. S. Sokolova[c], and V. N. Krylov[a]

[a] *Scientific Center, Research Institute for Genetic and Selection of Industrial Microorganisms, Moscow, 117545 Russia*
e-mail: krylov@genetica.ru

[b] *Shubnikov Institute of Crystallography, Russian Academy of Sciences, Moscow, 119333 Russia*
e-mail: eugenia.pechnikova@gmail.com

[c] *Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, 119991 Russia*
e-mail: sokolova184@gmail.ru

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Abstract—The properties of new virulent bacteriophage TL of *Pseudomonas aeruginosa* belonging to the family Podoviridae (genome size of 46 kb) were investigated. This bacteriophage is capable of lysing the bacterial lawn in halo zones around negative colonies (NC) of other bacteriophages. TL forms large NC, that are hardly distinguishable on the lawn of *P. aeruginisa* PAO1. At the same time, on the lawns of some phage-resistant PAO1 mutants, as well as on those produced by a number of clinical isolates, TL forms more transparent NC. It is suggested that more effective growth of the bacteriophage TL NC is associated with the differences in outer lipopolysaccharide (LPS) layer of the cell walls of different bacterial strains, as well as of the bacteria inside and outside of the halos. This TL property was used to optimize selection of bacteriophages producing halos around NC on the lawn of *P. aeruginosa* PAO1. As a result, a group of bacteriophages differing in the patterns of interaction between their halos and TL bacteriophage, as well as in some characters was identified. Taking into consideration the importance of cell-surfaced structures of *P. aeruginosa* in manifestation of virulence and pathogenicity, possible utilization of specific phage enzymes, polysacchadide depolymerases, for more effective treatment of *P. aeruginosa* infections is discussed.

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INTRODUCTION

Uncontrolled use of antibiotics for the treatment of bacterial infections promoted the appearance of pathogenic bacteria showing multiple resistance to antibiotics. This situation stimulated the search for new antibiotics, as well as the new treatment methods, including the worldwide interest to phage therapy. This term currently means not only the use of the mixture of living virulent phages, but also the use of phage-produced products, like lytic enzymes and bacteriocins. The enzymes, controlled by phage genome and able to destruct the components of the bacterial outer membrane and capsule, exopolysaccharides and lipopolysaccharides, which determine attachment of the bacteria to surface glycoproteins of the macro-host cells, can be also used for these purposes. Reacting with the antibodies at a distance from cell surface, lypopolysacharides (LPS) protect outer and cytoplasmic membranes of the bacterial cells from the destructive effect of complement. Free exopolysaccharides coat the cells thereby protecting them from the access of antibiotics. These factors substantially decrease effectivity of the macro-host defense systems, as well as the effectivity of antibiotic therapy.

For many bacteriophages, the bacterial cell wall LPS serve as adsorptional receptors [1–3]. During coevolution of the bacteria and phages, adaptation of phage polymerases to the destruction of exactly bacterial polysaccharides, took place. The practice of phage depolymerases degrading the LPS and exopolysaccharides is expected to reduce the resistance of pathogenic microorganisms to the action of different antibacterial factors and the stability of the microbial defense systems. Thus, specific bacteriophages represent an ideal source for producing depolymerases able to destruct the polysaccharide matrix of pathogenic bacteria.

Production of phage polymerases is (albeit not necessarily) manifested as a halo of the bacterial lawn clearing around NC. The halo-producing bacteriophages were described in many bacterial species, including *P. aeruginosa*. Depolymerase encoded by the *P. aeruginosa* infecting phage, which was the component of the phage particle and involved in the phage adsorption was described [4, 5]. Halos are formed as a result of enzymatic degradation of mucoid structures on the surface of bacterial cells after the lysis of infected bacteria. Bacterial lysis is accompanied by a release of the products a plenty synthesized during

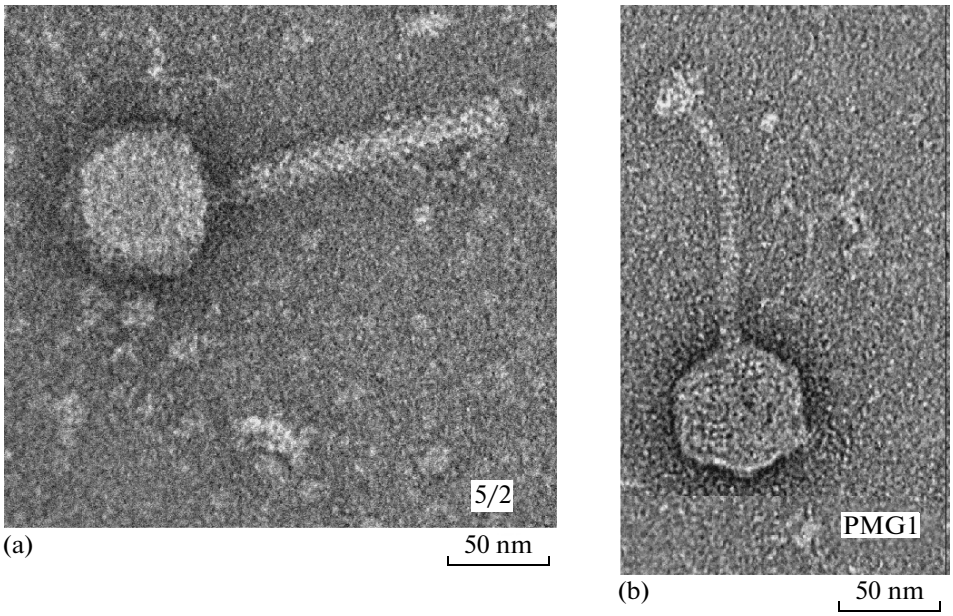

Fig. 1. Particle morphology of phages producing halos around NC on the lawn of *P. aeruginosa* PAO1. (a) Bacteriophage φ5/2 (Myoviridae); (b) bacteriophage PMG1 (Syphoviridae).

vegetative phage development. These can be, for example, LPS depolymerases, either in free, or structured form (the components of phage tail plates). Purified enzymatic preparations affected the lawn of grown bacteria, forming halos [4] due to the destruction of outer bacterial coat LPS without causing the death of bacterial cells. It was demonstrated that in case of *P. putida* halo production actually was not associated with bacterial death, and could be caused by the activity of depolymerases with different specificities [6].

In our experiments with bacteriophages of *P. aeruginosa*, the bacteriophage designated as TL and specifically affecting (clearing) halos formed by some phages active on *P. aeruginosa* PAO1, was isolated. The clearing occurred provided easily identification of bacteriophages forming even narrow or weakly manifested halos.

MATERIALS AND METHODS

Experiments were carried out using the *Pseudomonas aeruginosa* strain PAO1 (kindly provided by B. Holloway, Australia) and its mutants selected earlier [7], as well as in the course of the present study. Clinical isolates of *P. aruginosa* (kindly provided by Prof. M. Vaneechoutte (Belgium) and Dr. M.A. Popova (Chelyabinsk).

The search for halo-producing phages was carried out among the phages from our laboratory collection, mostly among the phages classified earlier [8]. This group consisted of 16 phages, including the phages isolated by us (L0, PMG1, 297, 21a, PMN22, 5/2, RAO3, SL2, SL, and PC11-2) and those obtained from other sources. Phages D3, F116, and G101 were kindly provided by B. Holloway, Australia; phage Sm was obtained form Dr. Yu.D. Tsygankov; phage DKB2 was provided by Dr. N.N. Sykilind. Bacteriophage TL was isolated in our laboratory.

Traditional methods of phage cultivation, analysis, and classification were used (restriction analysis of phage DNA, evaluation of the genome homology, and electron microscopy) [9–12].

Preliminary classification of the phages, previously not included in the procedure of standard classification, was carried out using the method based on comparative analysis of the phage growth on the group of bacterial phage-resistant mutants with consideration of formal scheme of adsorptional receptors [7].

Specificity of phage capsule polysaccharide polymerases were compared using the method described in [13]. Using this method, dispersal of halos formed on the control strain to the adjacent lawn of the tested strain was examined.

RESULTS AND DISCUSSION

Selection of Halo-Producing Bacteriophages of P. aeruginosa Through the Interaction with Bacteriophage TL

Specific features of bacteriophage TL (see below) made it possible to distinguish 16 phages producing halos around NC on the bacterial lawn among the laboratory collection of phages active on *P. aeruginosa* (about 200 specimens). According to electron microscopic data (Fig. 1), most of these phages belonged to the family Syphoviridae (Fig. 1b). Two phages produc-

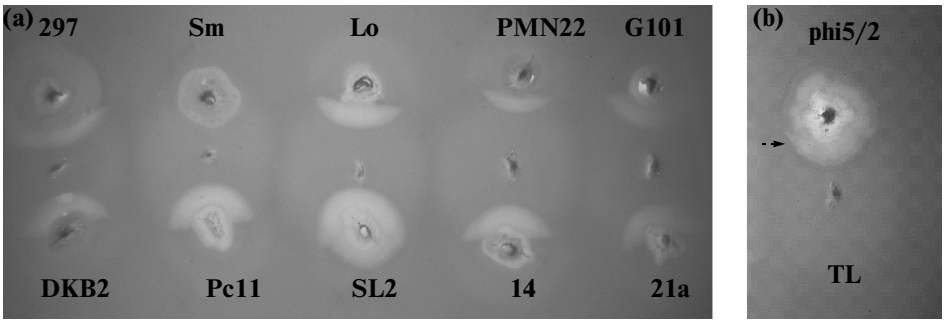

Fig. 2. Interaction between bacteriophage TL and bacteria on the lawn of *P. aeruginosa* PAO1 in the halos around NC of some bacteriophages. (a), Transparent regions in the overlapping zone between halos and NC of phage TL, and the absence of interaction with halo of phage SM. (b), narrow, clearer band (indicated by an arrow) in the halo of phage 5/2 in the contact zone with NC of phage TL.

ing halos around NC belonged to the species PB1 (family Myoviridae) (Fig. 1a), Based on comparison of DNA restriction profiles obtained with the phages, these phages were attributed to six species according to the sensitivity to different restriction enzymes. The phages selected differed in exteriority of the halos and in the time needed for their development. Some of the phages produced clearly visible halos as early as on the second day of incubation, while the others produced halos on the third and fourth days. Halo identification strongly depended on the conditions of cultivation, including the medium batch, thickness of agar medium layer, bacterial host used, etc. That was the use of bacteriophage TL, which speeded up the work and made it possible to overcome this uncertainty. It was demonstrated that TL specifically interacted with different phages in the halo zone. It was suggested that the reason underlying the interaction between the NC of phage TL and those of halo-producing phages was the same as that revealed during the analysis of the differences of the TL growth patterns on different bacterial lawns. For instance, NC formed by TL on the lawn of the PAO1 bacteria were very turbid and often almost indistinguishable from the lawn. The sizes of these NC constituted about 5 mm in diameter. At the same time, on some mutants of this strain, as well as on a number of clinical isolates the TL growth was accompanied by the formation transparent NC of two morphological types. In case of the contact of mutant NC of phage TL, growing on the PAO1 lawn near the NC of putative halo-producing phage, distinct clearing in the overlapping zone was observed. In the cases of unclear halo manifestation, the use of phage TL made it possible to confirm either halo existence, or the absence of halo sensitivity to this phage. Thus, three types of interactions between the TL and halos of different phages selected were distinguished (Fig. 2). For instance, halo of phage SM had no reaction to the NC of phage TL. On the other hand, phages 5/2 and BP1 produced no visible halos after two to three days of incubation, but displayed reaction to TL in the form of narrow, more clear band in the contact zone between two NC. Finally, phages of the third type produced visible halos, inside of which the growth of phage TL led to the formation of transparent lysis zones. Thus, the unique feature of phage TL is specific activation of its growth upon infection of the bacteria located inside the NC halos of certain bacteriophages. It seems likely that improvement of the TL growth occurs as a result of structural modification of bacterial cell surface in the halos due to the action of specific phage LPS depolymerases.

Investigation of Phage TL Properties

Taking into consideration unusual properties of phage TL, its detailed examination was performed. Although phage TL produces very turbid NC on the lawn of *P. aeruginosa* PAO1, infection of these bacteria does not lead to lysogen formation. Based on the phage particle electron microscopic data, phage TL was assigned to the family Podoviridae. It has a 59-nm icosahedric head and a tail with the length about 42.5 nm (Fig. 3). The data of restriction analysis indicated that the TL genome size was about 46 kb (Fig. 4). Bacte-

Comparison of the phage φPLS27 and TL DNA samples by the number of restriction sites of different endonucleases

Endonuclease	No. of fragments	
	φPLS27*	TL
Sal I	3	–
Hind III	–	>10
Bam HI	–	6
Xho I	–	3
Eco RI	–	>11
Sma I	2	–
Pvu II	4	9
Genome size, kb	42.5	46.2 (*Bam* HI)

* Elicited from [14].

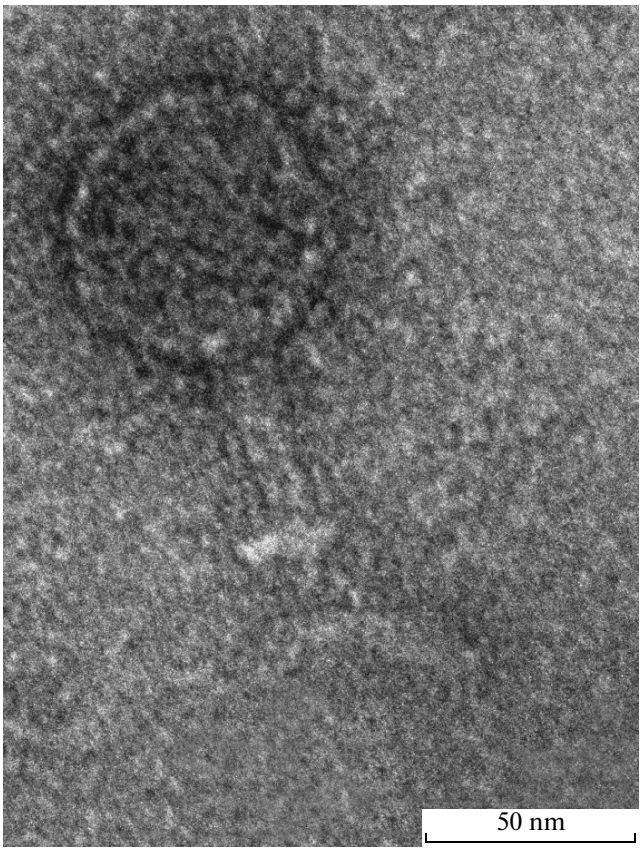

Fig. 3. Particle morphology of phage TL.

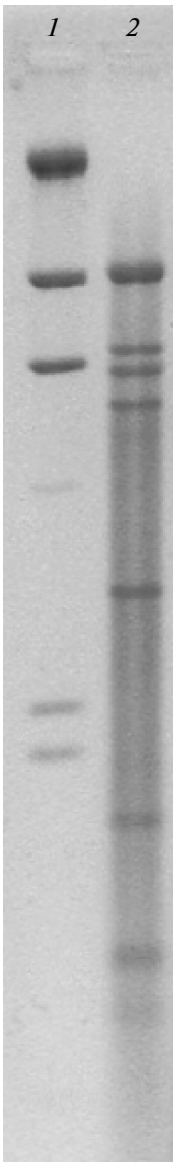

Fig. 4. Electrophretic separation of the TL DNA fragments digested with the *Hind*III endonuclease. 1, DNA λ *Hind*III; 2, DNA TL *Hind*III.

riophage TL displays formal similarity to phage φPLS27, capable of growth on only rough colonies, deprived of external LPS layer [14]. However, phages TL and φPLS27 are substantially different in sensitivity of their genomes to different endonucleases (table).

We have isolated a number of *P. aeruginos*a PAO1 mutants, where TL produced on PAO1, grows with the development of NC of two morphological types (absolutely clear and more turbid). Both NC types have similar size of 5 mm. One of these types is more stable, while another one permanently forms NC of both types. However, plating on *P. aeruginosa* strain these variants are indistinguishable by external appearance, as well as by the interaction effect with the bacteria in the halo zones of halo-producing phages.

Since TL is a virulent phage capable of lysing the bacteria resistant to a number of other phages, the use of TL as a component of phage therapeutic mixtures after sequencing and genome annotation seems to be promising.

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