

**DEPENDENCE BETWEEN ACYLATION DEGREE
AND SPECIFIC LYSIS ACTIVITY OF THE
PSEUDOMONAS AERUGINOSA M6
BACTERIOPHAGE**

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In recent years, a scientific renaissance has noted in the studies of bacteriophages, phenomena for the issues solution of theoretical and applied value [1].

Bacteriophages (phages) – bacterial viruses – have again become a favored subject for researching general biological problems, as an unrivalled instrument in molecular biology (including the studying of gene activity regulation of molecular mechanisms), and general and special virology and ecology, with a spotlight on the role of phages in the life activities of natural and artificial microbial ecosystems [2]. The applied work researches the use and value of bacteriophages in the medical, industrial, agricultural and other applications of microbiology [3]. A traditional scientific area is the application of bacteriophage models in experiments for studying receptor interaction mechanisms, the kinetics of bonding protein molecules to cells target, and the raised affinity and special activity of immune-biological drugs [4].

The purpose of this work is to study the influence of chemical modification (various levels of protein acylation) on the level of lytic activity and other biological properties of *Pseudomonas* M6 (PM6) [5]. Earlier, analogous studies were performed on the modified *Klebsiella* 45 bacteriophage [6].

Materials and Methods

The subject of the study are samples of *Pseudomonas* O 12 bacteriophage (hereinafter named the phage or the M6 phage) [7], which, like the strains of bacteria used in this work, were obtained from the Microorganism Museum of the State Science and Control Institute of Biotechnology and Microorganism Strains (Kiev, Ukraine).

The source of PM6 is lysogenic strain No. 969 of *Pseudomonas aeruginosa* in the National Collection of Type Cultures in the Central Public Health Laboratory, London (NCTC) [8]. The indicator strain is *Pseudomonas aeruginosa* ATCC 9027 (O-2, Fisher-Devlin immunotype 3) ATCC [9].

Generally accepted methods, as described in handbooks, were used in working with the phages [10, 11, 12, 13]. Pure PM6 was obtained from individual standard-morphology phage plaques (plaque-forming unit, or PFUs), after ten passes of the phage over the indicator strain [14]. In order to prepare purified PM6 preparations

with a high phagolysate titer on an agarized meat-peptone culture medium, they were processed with chloroform (volume mix ratio: 1:1) for ten minutes and then the phages were desalted with a saturated solution of ammonium sulfate and a subsequent solution of the precipitate, along with a cold dialysis for 48 hours. The purification of the preparations and the calibration of the PM6 by size were carried out using the gel filtration method on Sephadex G-75 (2 x 50 cm column). To dilute the phage suspension, a TM buffer was used (10 mM tris HCl with a pH of 7.5, 10 mM MgSO₄). The purity (homogeneity) of the PM6 preparations was monitored via electron microscopy (UEMV-100B electron microscope, negative contrast using 2% solution of phosphotungstic acid with a pH of 7.0, 50,000-200,000x) and the immunotechnique in neutralization reactions with antiphage (against M6 phages) and antibacterial (against the *P. aeruginosa* M6 strain) serums, obtained from hyperimmunised rabbits. In subsequent experiments, PM6 samples were selected that had a sufficient level of purity (homogeneous in the morphology of virions that inactivated the homologous antiphage serum by no less than 99.0% and the heterologous antibacterial serum by no more than 1%) and a titer no lower than 10 PFU per ml (PFU/ml). Titration of the PM6 conducted using the standard bilayer agar method [15]. The crude protein content in PM6 suspensions was determined using the spectroscopic method (at 280 and 260 nm) as per Barburg and Christian [16]. The resultant PM6 samples were diluted with a TM buffer to a final protein concentration of 1 mg/ml, which approximately corresponded to the phage titer of 10⁹ in PFU/ml. Succinylation of the PM6 samples were conducted according to the method developed by T.J. Molenaar [17]. The amount calculation of the succinic anhydride for PM6 succinylation with different values, from 5%, 10%, 20%, up to 100% were made as established in [18]. The mixture was agitated in a shaker until the full dissolution of the anhydride, and the pH brought to 7.5 through the addition of 6 M of NaOH solution.

The influence of the acylation PM6 proteins on the interaction process between the phage and the bacterial cell was studied in experiments for the determination of adsorption speed constants, latent period duration, and average yield per cell during the study of a phage single cycle development with corrections to time intervals, when these studies were conducted using *Pseudomonas* phages [19]. The number of bacterial cells was determined in colony-creating units (CCU). The PM6 lytic activity spectrum was determined through spot tests at 224 strains *Pseudomonas* genus and 106 other genera strains (correspondingly, *Enterobacter* -40, *Escherichia* -10, *Citrobacter* -8, *Hafnia* -7, *Serratia* -6, *Shigella* -10, *Salmonella* -8, *Proteus* -7) through the use of PM6 suspension containing 10⁵ - 10⁶ PFU/ml. (This concentration of infectious phage corpuscles corresponds with the critical distribution of the M6 phage) [20, 21; 22].

The results of the research were subjected to statistical processing using single-factor dispersion analysis [23].

Results and Discussion

The *Pseudomonas aeruginosa* M6 bacteriophage (PM6) is a typical phage (corresponds in T-pair structure with *E. coli* phages), and according to virion morphology, it belongs to D. Bradley's A group [24]. The measurements ($m \pm d$) of the M6 phage corpuscle components are the following: equilateral heads has isometric polygon form: 65 ± 9 nm; intact tail length and width: 145 ± 7 and 12 ± 2 nm respectively; shortened tail length and width: 38 ± 4 and 18 ± 2 nm respectively; shaft tail width: 6.0 ± 0.5 nm with an axial channel of 3.0 ± 0.5 nm. The tail sheath does not cover the entire shaft, leaving part of it – the “neck” near the head – exposed. Transverse striping with an interval of 2-2.5 nm distinguished on the intact sheath surface. The sheath's distal end has a well-expressed basal plate with projections, which is wider than the tail's diameter. On tails with shortened sheaths, the basal plate stretched up to the phage's head. In empty heads, electron-dense plates in the form of a flap is seen in the place where the tail attaches. Fibrils (threads) protrude from the basal plate. In a significant number of the phage corpuscles, the threads are twisted into bunches. It is these structural elements, which allotted for the role of an organ for special absorption. The PM6 adsorption apparatus is the most sensitive for lytic activity and the first target for the chemical modification [25].

In electronic microscopic studies of acylated PM6 (with 5%, 10%, 20%, and 100% protein acylation levels), morphological changes were not discovered. The virions' measurements and structures were all the same type and the characteristics did not vary from those in the intact PM6. The single morphology of the PM6 samples studied is clearly not only the result of cloning when a pure line is obtained, but is a consequence of the previous (pre-acylation) PM6 calibration by size through gel filtration on Sedaphex G-75. This methodological approach is also beneficial for increasing the precision PM6 acylation level, [26]. Without conducting additional PM6 calibration, the statistical error for determining their acylation level by this method is $\pm 3\%$ [Ошибка! Закладка не определена.]. However, it is well-known that the amino acid makeup of the PM6 proteins from each structural element (head, tail, basal plate, and so on) are specific and different. Therefore, it can be said, that in actual experimental conditions, the proteins' chemical modification level by each structural element from PM6 is substantially different. A precise determination of the real chemical modification level of each virion's structural component is still methodologically difficult (including cases in which computer molecular modeling is used), which causes scientists to actively use an empirical approach in the search for chemical modifiers for obtaining the desired changes to the properties of the objects studied.

In our experiments, PM6 lytic activity was preserved and its antigenic characteristics did not change after acylation. Homologous antiphage serums (to treat intact and chemically modified PM6) retain activity at 95-99% level for neutralization various PM6 sample; also a neutralization speed constant (with a serum exposure processing time of 10 min) is $67.7 \pm 15.0 \text{ min}^{-1}$. The neutralization reaction for intact and acylated PM6 by heterologous antiphage serums was 23 and 40 for the same type. Processing using these serums led to a loss of the level capable to form plaques in the samples by $(4.4 \pm 1.8)\%$ and $(81.1 \pm 7.7)\%$ respectively.

This is supported by the fundamental research data. That the neutralizing antibodies from antiphage serums act on PM6 antigens localized to the tail (the zone of irreversible bond formation), disrupting the receptor apparatus and preserving the PM6 specific adsorption ability.

Also acylated derivatives of the phage M6 (ADPM) formed plaques: the size of the lysis zone was 1.0-1.5 nm, the borders of the zone were even and well-defined, and the entire zone was transparent (incomplete lysis zones were not formed). The lytic activity of the ADPM are not different from the initial sample. The intact PM6 has a working titer 175 (55.8%) for 112 *P. aeruginosa* strains. Four strains of *Enterobacter* from 40 (10.0%) were sensitive to the PM6. ADPM with modification degrees of 5%, 10%, and 20% had a wider spectrum of lytic activity, up to 56.7%, 57.1%, and 56.7% respectively. In *Enterobacter* cultures, ADPM has the same lytic activity spectrum as intact PM6. All of the ADPM retained a high level of specificity in their lytic action, just as its output samples did not lose a single strain of the bacteria in the *Escherichia*, *Citrobacter*, *Hafnia*, *Serratia*, *Salmonella*, *Shigella*, and *Proteus* genera.

A certain dependence has been noted between the acylation level of the ADPM in general and the level (titer) of specific lytic activity in the M6 phage (Table 1). This dependence is not a simple linear function; therefore, the change in the succinylation level is accompanied by both an increase and a decrease in its activity titer.

The highest ($p < 0.05$) increase in activity was noted in the 5% acylation level. An increase in the level of acylation by 10% and 20% was accompanied by a less-intensive ($p < 0.05$) increase in the titer activity of the ADPM (by a factor of 12 and 8 respectively), in comparison with the initial titer. A 100% percent succinylation decreased the activity of ADPM used in the experiment by more than 10^3 times. The ADPM retained an 80-95% lytic activity if determined at the storage along one year (observation time) in hermetically sealed glass containers at $t = +6 - +8^{\circ} \text{C}$. Analogous storage patterns were observed for non-modified phage.

The dependence between the acylation level and ADPM adsorption on the bacterial cells presented in Table 2.

The data from Table 2 indicate that ADPM has substantially increased the level of some interaction parameters ($p < 0.05$), like adsorption speed (the adsorption speed constant increased by 8-9 times), latency period duration (reduced to 5 minutes: minimal methodically determined time interval). It should be noted that the adsorption speed constant increased, while the duration of the latency period decreased in a nearly identical amount ($p > 0.05$), independent of the acylation level. Other researchers have voiced similar hypotheses [Ошибка! Залка не определена.] after studying the patterns of adsorption, endocytosis and reproduction of a standard M13 bacteriophage and its chemical modifications using galactose and succinic acid (lacM13 and sucM13 respectively) under *in vivo* conditions (in mice). It has been demonstrated that M13 phage succinylation leads to an increase in affinity to the target receptors due to a change in the surface charge while the antigen structure remains relatively well-preserved. In another study [Ошибка! Залка не определена.] it shows that succinylation of

a human serum albumin leads to an increase in its affinity to the viral adhesins.

On the PM6 models, it has been proven that various acylation protein levels changed the level of phages specific lytic activity and influence on the interaction parameters with sensitive bacterial cells (the adsorption speed content and latent period duration). It was proven that ADPM (with 5%, 10%, 20%, protein acylation levels) increased specific lytic activity at ninety, twelve, and eight respectively. The adsorption speed constant increased by nine, eight, and eight respectively. In addition, the ADPM duration latent period decreased by 20% in all cases. It is notable that ADPM with a 100% acylation level has adsorption on sensitive cells and is accompanied by a significant decrease in specific lytic activity (more than 10^3 times). The process of chemical modification (succinylation) does not cause changes to the morphology or antigen structure of PM6 virions and does not substantially influence the spectrum of lytic activity and average production of the PM6 infectious corpuscles.

Table 1. Lytic activity of ADPM against *P. aeruginosa subsp.*

Phage Specimen M6 phage, acylation level, %	Lytic Activity Titer, PFU/ml (m±d)
0	$(1.2 \pm 0.3) \cdot 10^8$
5	$(8.3 \pm 4.7) \cdot 10^9$
10	$(1.1 \pm 0.7) \cdot 10^9$
20	$(7.7 \pm 2.4) \cdot 10^8$
100	$< 10^5$ (titer test limit)

Table 2. *P. aeruginosa subsp.* and ADPM interaction parameters

Phage Specimen M6 phage, acylation level, %	Indicator of Phage Interaction with Bacterial Cell*	
	Adsorption Speed Constant (10^{-10} ml/min)	Duration of Latent Period (min)
0	6.3 ± 0.7	25-30
5	55.8 ± 8.3	20-25
10	53.3 ± 6.2	20-25
20	52.6 ± 8.2	20-25
100	Does not adsorb	Not specified

* $P \leq 0.05$ for single-factor dispersion analysis, comparison group: non-acylated phages

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Introduction

The purpose of this work is to study the influence of various degrees of protein's acylation on lytic activity and other biological properties of *Pseudomonas M6*

bacteriophage. **Materials and methods.** The subject of the study were samples of the *Pseudomonas O 12* bacteriophage, hereinafter named the M6 phage. Pure phage lines were obtained from individual standard-morphology phage plaques (plaque-forming units, or PFUs) after ten passes over the indicator strain. In subsequent experiments, M6 phage samples were selected that had a sufficient level of purity (homogeneous in the morphology of virions that inactivated the homologous antiphage serum by no less than 99.0% and the heterologous antibacterial serum by no more than 1%), and

a titer no lower than 10 PFU per ml (PFU/ml). Titration of the phages was conducted using the standard bilayer agar. The crude protein content in the phage suspensions was determined using the spectroscopic method (at 280 and 260 nm). Succinylation of the phage samples was conducted according to the method developed by T.J. Molenaar. The phage's lytic activity spectrum was determined through spot tests on 224 strains of the *Pseudomonas* genus and 106 strains of other genera (correspondingly, of strains *Enterobacter* -40, *Escherichia* -10, *Citrobacter* -8, *Hafnia* -7, *Serratia* -6, *Shigella* -10, *Salmonella* -8, *Proteus* -7) through the use of a phage suspension containing $10^5 - 10^6$ PFU/ml. (This concentration of infectious phage corpuscles corresponds with the critical distribution of the M6 phage). The results of the research were subjected to statistical processing using single-factor dispersion analysis. **Results and discussion.** One hundred percent phage protein acylation leads to a loss of adsorption ability on sensitive cells and is accompanied by a significant (more than 10^3 times) decrease in the level of specific lytic activity in the phage preparations. The succinylation type being studied does not cause changes to the morphology or antigen structure of phage virions

and does not substantially affect the lytic activity spectrum or the average yield of infectious phage corpuscles on a single sensitive bacterial cell. Considering the relative uniqueness of any biological object (including the M6 phage being studied), the authors recognize the advisability of verifying the established patterns of the effect of various acylation levels on the biological properties of other types and morphological groups of viruses. It was proven that at phage sample acylation levels of 5%, 10%, and 20%, the specific lytic activity increased by a factor of ninety, twelve, and eight respectively. The adsorption speed constant increased by a factor of nine, eight, and eight respectively. And the duration of the latent period fell by 20% in all cases with the development of a productive infection caused by the acylated samples of the phages. The use of this chemical modification method does not cause changes to the morphology or antigen structure of phage virions and does not substantially affect the average yield of infectious phage corpuscles on a sensitive bacterial cell. **Key words:** acylation, adsorption speed, lytic activity, *Pseudomonas* M6 bacteriophage.

References

- [1] Campbell A. The future of bacteriophage biology/ A. Campbell // *Nat.Rev. Genet.*- 2003.-Vol. 6., No. 4.- P. 471-477.
- [2] Gvozdyak R. I. Plant, phage, bacterium: a new hypothesis on their interrelation/ R. I. Gvozdyak // *Mikrobiol. zhurn.* - 1993. -Vol. 55., No. 6.- P. 92-94.
- [3] Sankaran N. The bacteriophage, its role in immunology: how Macfarlane Burnet's phage research shaped his scientific style/ N. Sankaran // *Stud Hist Philos Biol Biomed Sci.*- 2010.- Vol. 41., No. 4.- P. 367-375.
- [4] Kung V.L., Ozer E.A., Hauser A.R. The accessory genome of *Pseudomonas aeruginosa*/ V.L. Kung, E.A. Ozer, A.R. Hauser // *Microbiol Mol Biol Rev.*- 2010. -Vol.74, No. 4.- P.621-641.
- [5] Ceysens P.J. The genome and structural proteome of YuA, a new *Pseudomonas aeruginosa* phage resembling M6// P.J. Ceysens, V. Mesyanzhinov, N. Sykilinda Briers Y, et al.// *J Bacteriol.* 2008 Feb;190(4):1429-35.
- [6] Martynov A.V. Effect of succinylation of Klebsiella 45 bacteriophage proteins on specific lytic activity/ A.V. Martynov, O.M. Tymchenko, S.I. Pokhyl // *Mikrobiol Z.*- 2005.- Vol.67, N. 5.- P.80-88.
- [7] Pitta T.L., Livermore D.M., Pitchera D., Vatopoulou A.C. and Legakisa N.J. Multiresistant serotype O 12 *Pseudomonas aeruginosa*: evidence for a common strain in Europe/ T.L. Pitta, D.M. Livermore, D. Pitchera, et al. // *Epidemiology and Infection.*- 1989.- N.103.-P. 565-576
- [8] Kwan T. Comparative genomic analysis of 18 *Pseudomonas aeruginosa* bacteriophages/ T. Kwan, J. Liu, M. DuBow et al. // *J. Bacteriol.*- 2006.- Vol.188, N. 3.- P. 1184-1187.
- [9] Zhang Y. Enhanced octadecane dispersion and biodegradation by a *Pseudomonas rhamnolipid* surfactant (biosurfactant)/ Y. Zhang, R.M. Miller // *Appl. Environ. Microbiol.*- 1992.-Vol. 58. 3276-3282
- [10] Adams M.H. Bacteriophage manipulations, pp. 19-22. In *Bacteriophages*. Interscience Publishers, Inc.- 1959., New York, NY.
- [11] Goldfarb A. Control of promoter utilization by bacteriophage T4-induced modification of RNA polymerase alpha subunit/ A. Goldfarb, P. Palm // *Nucleic Acids Res.*- 1981.-Vol. 9, N. 19.-P.4863-4878.
- [12] Kleckner N. Genetic engineering in vivo using translocatable drug-resistance elements. New methods in bacterial genetics/ N. Kleckner, J. Roth, D. Botstein // *J Mol Biol.*-1977. - Vol.116, N. 1.- P.125-159
- [13] Josko D. Molecular methods in clinical microbiology. Introduction / D. Josko // *Clin Lab Sci.*- 2010.- Vol.23, N.4.- P.228-230
- [14] Górski A. Bacteriophage therapy for the treatment of infections/ A. Górski, R. Miedzybrodzki, J. Borysowski, et al. // *Curr Opin Investig Drugs.* -2009.- Vol.10, N 8.- P.766-774.
- [15] Viazis S. Isolation and characterization of lytic bacteriophages against enterohaemorrhagic *Escherichia coli*/ S. Viazis, M. Akhtar, J. Feirtag, et al. // *J Appl Microbiol.*- 2011.- Vol.110, N. 5.-P.1323-1331
- [16] *Practical Chemistry of Protein*, Ed. A. Darbe., Moscow (1989)
- [17] Molenaar T.J. Uptake and processing of modified bacteriophage M13 in mice: implications for phage display / T.J. Molenaar, I. Michon, S.A. Haas et al. // *Virology.* -2002. -Vol.1, N 293. -P.182-191.

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- [18] Kuipers M.E. Mechanism of anti-HIV activity of succinylated human serum albumin / M.E. Kuipers, M. vd Berg, P.J. Swart et al.//Biochem. Pharmacol. -1999. – Vol.8, N 57.-P.889-898.
- [19] Pini A. Antimicrobial activity of novel dendrimeric peptides obtained by phage display selection and rational modification/ A. Pini, A. Giuliani, C. Falciani, et al. // Antimicrob Agents Chemother.- 2005.- Vol.49, N. 7.- P.2665-2672.
- [20] Smith G. P. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface/ G. P. Smith // Science.-1985.- N.228.- P.1315-1317.
- [21] Morello E. Pulmonary Bacteriophage Therapy on *Pseudomonas aeruginosa* Cystic Fibrosis Strains: First Steps Towards Treatment and Prevention/ E. Morello, E. Saussereau, D. Maura, et al. // PLoS One.- 2011.- Vol.6, N. 2.-e16963.
- [22] Kay M.K. Bacteriophage ecology in *Escherichia coli* and *Pseudomonas aeruginosa* mixed-biofilm communities/ M.K. Kay, T.C. Erwin, R.J. McLean, G.M. Aron // Appl Environ Microbiol.- 2011.- Vol.77, N. 3.- P.821-829.
- [23] Januszyk M. Statistics in medicine/ M. Januszyk, G.C. Gurtner // Plast Reconstr Surg.- 2011.- Vol.127, N. 1.- P.437-444.
- [24] Bradley D. The morphology and physiology of bacteriophages as revealed by the electron microscope / D. Bradley // J. Roy.Microscop.Soc. -1965. – N. 84. - P.257-316.
- [25] Frist R.H. The protein subunit of cucumber virus 4; degradation of viruses by succinylation/ R.H. Frist, I.J. Bendet, K.M. Smith, M.A. Lauffer // Virology.- 1965.- Vol.26, N. 4.-P.558-666
- [26] Ackermann H. W., Cartier C., Slopek S., Vieu J. F. Morphology of *Pseudomonas aeruginosa* typing phages of the Lindberg set/ H. W. Ackermann, C. Cartier, S. Slopek, J. F. Vieu // Ann. Inst. Pasteur Virol.- 1988.- N.139.- P.389-404.