Microbiology, Vol. 71, No. 1, 2002, pp. 72–77. Translated from Mikrobiologiya, Vol. 71, No. 1, 2002, pp. 82–88.

EXPERIMENTAL ARTICLES

A Study of *Erwinia carotovora* Phage Resistance with the Use of Temperate Bacteriophage ZF40

F. I. Tovkach[1]

Zabolotnyi Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, ul. Zabolotnogo 154, Kiev, Ukraine

Received December 26, 2000; in final form, May 7, 2001

Abstract—The causes of the unique phage resistance of the pectinolytic phytopathogenic strains of *Erwinia carotovora* were studied with the use of temperate bacteriophage ZF40. It was shown that, in these bacteria, the bacteriophage–cell interaction can be substantially blocked at the adsorption level. An adequate indicator for studying the temperate bacteriophages of erwinias was developed on the basis of mutants resistant to macromolecular bacteriocins. Various restriction–modification systems, which influence cell resistance to bacteriophages, were revealed for the first time in *E. carotovora*. The phage resistance was shown to be determined by the wide occurrence of homoimmune temperate viruses in pectinolytic erwinias.

Key words: Erwinia carotovora, phage resistance, temperate bacteriophage, lysogens.

At present, the phenomenon of bacteriophagia in *Erwinia carotovora*, as distinct from other bacteria, has not been adequately explored [1, 2]. So far, the descriptions of erwinial bacteriophages are sporadic [3, 4]. The reasons why erwiniophages occur in natural environments less frequently than the phages of other enterobacteria are far from being completely understood [1, 2].

The aim of this work is to study the unique phage resistance of pectinolytic phytopathogenic erwinias using the temperate bacteriophage ZF40, which I revealed when studying the bacteriocinogenic properties of a large group of *E. carotovora* strains [5].

MATERIALS AND METHODS

The study of the host range of bacteriophage ZF40 used the collection set of strains of *Erwinia carotovora* subsp. *carotovora* (ECA) and *E. carotovora* subsp. *atroseptica* (EAT) of the Department of Microbiology, Belarus University (Prof. Yu.K. Fomichev); the complete list of strains was published earlier [6]. Three ECA strains—62A, 33A, and M2-4—were most frequently used in the study, as well as strain *Erwinia* sp. ZM-1. Twenty-nine different macromolecular carotovoricins (MCTV) able to kill the cells of ECA 62A were used in tests [5, 7]. The virulent bacteriophage T7M, Meselson's variant (henceforth T7), was provided by T.P. Pererva (Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine). The bacteriophage was obtained from a separate negative colony at a concentration of $4\,4 \times 10^{11}$ PFU/ml on *Escherichia coli* BE.

The bacteria were grown on liquid and solid LB medium. Phage ZF 40 was obtained at concentrations of 1×10^{10} to 2×10^{11} PFU/ml by the confluent lysis method. The study of temperate bacteriophages and lysogeny was carried out on the basis of conventional classical methods. The growth temperature for phage ZF40 was, as a rule, 2 to 3°C lower than the optimal temperature for the growth of *E. carotovora* strains and constituted 25°C.

The mutants of *E. carotovora*. subsp. *carotovora* 62A resistant to macromolecular carotovoricins (Ear9VII, EcaEc153, EcaM2-4, and Eca55A) were selected using the following original method. Negative plaques were obtained on the lawn of the initial strain by means of bacteriocin [5]. The cells that had survived the lysing action of MCTV were transferred to a liquid LB medium from the center of the negative plaque and grown on a shaker to attain a concentration of 5×10^8 to 7×10^8 cells/ml. The lawn formed by these bacteria was repeatedly treated with the same bacteriocin. After repeated treatments, the cells were plated to obtain separate colonies. Individual RC mutants (RC means resistance to carotovoricin) were subcultured twice on solid LB medium. Subsequently, the mutants were tested for sensitivity to the bacteriocin that had been used for their selection and for sensitivity to the other 29 MCTV, which lyse the initial strain ECA 62A. The ability to maintain the development of phages T7 and ZF40 was determined for the mutants. The growth of the initial strain ECA 62A and the RC-mutants was studied on solid LB medium and on LB with 0.4% sodium deoxycholate (SDC).

[1]E-mail: feodorit@mail.ru

0026-2617/02/7101-0072$27.00

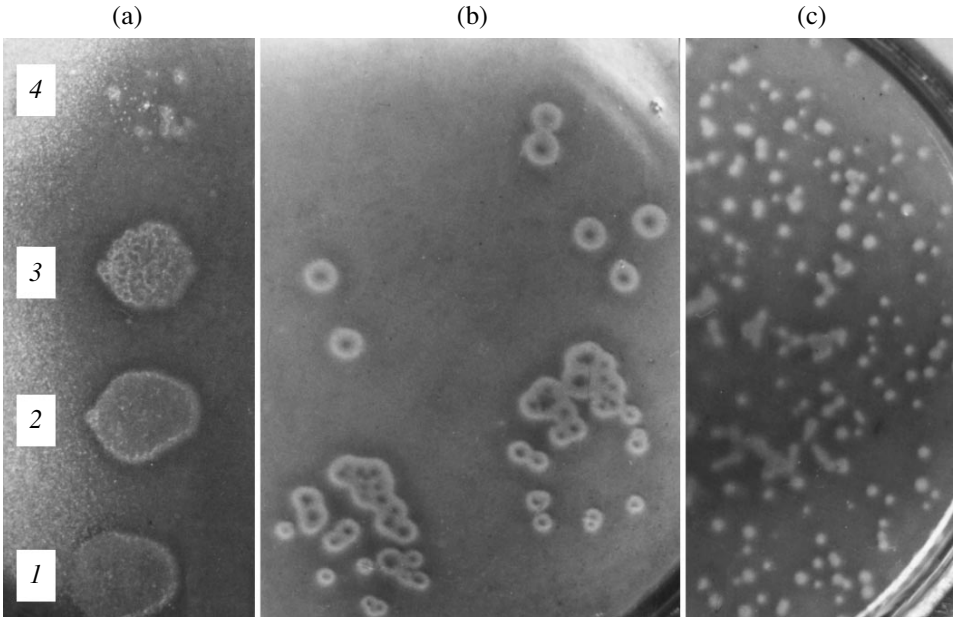

Fig. 1. Negative colonies of the temperate bacteriophage ZF40 on different indicator strains: (a) ECA 62A; (b) RC5297; (c) ECA M2-4. *1–4*, increasing tenfold bacteriophage dilutions.

The three-layer agar method was used for qualitative studies of bacteriocin and bacteriophage adsorption [8]; the concentration of phage particles was minimal and was determined empirically.

The efficiency of inoculation of sensitive cultures with phages T7 and ZF40 was expressed as a quantitative titer ratio. For phage T7, the control indicator was strain *E. coli* BE provided by I.I. Serysheva (Institute of Biochemistry, Russian Academy of Sciences).

The preparation of purified phage particles, the isolation of DNA, and its restriction analysis were described by us earlier [9]. Endonuclease *Hpa*I was used for the comparative analysis of phage genomes.

RESULTS AND DISCUSSION

Erwiniophage ZF40 was obtained when testing *Erwinia* sp. ZM-1 lysates, induced with mitomycin C, on the lawn of *E. carotovora* subsp. *carotovora* 62A [5]. Application of these lysates in an amount of 5 μl on the lawn of sensitive ECA 62A cells always results in the formation of several dozens of negative phage colonies. Multiple titration of lysates showed that the bacteriophage population was represented by two phage types. The first type forms small colonies (s-plaques) and prevails quantitatively over the second type producing large phage colonies (l-plaques). Pure phage lineages were obtained from both types of plaques by cloning. In so doing, I did not succeed in obtaining monotypic colonies for any pure phage lineage (Fig. 1a). Subsequently, the DNA of these phages, called ZF40-s and ZF40-l, was analyzed by hydrolysis with endonuclease *Hpa*I. By the distribution pattern of the restriction sites and by the molecular mass of the DNA fragments, the phage variants appeared to be identical. Thus, the heterogeneity of the primary phage population is not determined by the presence of contaminating bacteriophages; nor is it determined by considerable variations in the phage genome (large deletions or insertions).

Subsequently, the efficiency of phage ZF40-l reproduction was studied using the Eca 62A indicator culture. Both in liquid culture and when growing the phage by the confluent lysis method, the yields of viable particles reached 1×10^{10} PFU/ml. These results suggest that no intracellular restriction exists on phage ZF40 reproduction in strain ECA 62A. For further exploration of the cause of heterogeneity of the phage population, RC-mutants resistant to MCTV, Ear9BII, EcaEc153, EcaM2-4, and Eca55A were obtained. As can be seen from the data shown in Table 1, the carotovoricins allow the selection of resistant mutants with different properties. The use of Eca55A results in three different RC-mutants. All three resistant mutants are characterized by a drop in the survival rate when they are grown on solid LB medium with 0.4% sodium deoxycholate. For some mutants, the survival decreases to 0.05–0.02% (mutants RC5297 and RC5191), whereas this value for the parent strain ECA 62A constitutes approximately 45%. All the mutants show, to an extent, a decrease in sensitivity to 29 active carotovoricins that kill the parent strain with 100% efficiency. One of the mutants, RC5191, is absolutely resistant to the test MCTV. Three mutant types selected using the MCTV Eca55A acquired sensitivity to infection by the *E. coli* phage T7 (Table 1); the greatest inoculation efficiency (IE = 5×10^{-4}) was observed for mutant

Table 1. Characteristics of *E. carotovora* subsp. *carotovora* 62A mutants resistant to macromolecular carotovoricins

Mutant	Selective MCTV	Survival rate (%) in SDC	Sensitivity to MCTV (S-, %)	Phage inoculation efficiency	
				T7	ZF40
62A	–	45	100	0	1 (h)
RC5022	Ear9BII	12	96	0	1 (h)
RC5029		–	96	0	1 (h)
RC5293	EcaEc153	0.2	75	0	0.5 (s)
RC5297		0.05	75	0	0.9 (l)
RC5501	EcaM2-4	17	28	0	0.9 (l)
RC5195	Eca55A	20	38	1×10^{-5}	0
RC5192		–	38	1×10^{-4}	0
RC5191		0.02	0	5×10^{-4}	0

Note: "–" denotes that the parameters were not determined; "h," "s," and "l" signify heterogeneous, small, and large colonies, respectively.

RC5191. The opposite effect was obtained when titrating phage ZF40 on RC-mutants and the parent strain ECA 62A (Table 1). Bacteriophage ZF40 inoculates normally the mutants RC5022, RC5029, RC5293, RC5297, and RC5501 (IE = 0.5 to 1.0) but has a zero inoculation efficiency on three mutants, namely, RC5191, RC5192, and RC5195. Only on two mutants does bacteriophage ZF40 form large negative colonies with a well-discernible turbid center (Fig. 1b).

The analysis of the results presented suggests that one of the causes of *E. carotovora* phage resistance is blockage of bacteriophage ZF40 adsorption. The hampered phage–cell interaction is likely to be one of the main causes of the heterogeneity of negative colonies in the initial strain ECA62A (Fig. 1a). The diversity of mutants resistant to bacteriocins (no less than five different types, Table 1), as well as an increased sensitivity to sodium deoxycholate, indicates that the mutants are likely to have a changed structure of the lipopolysaccharide (LPS) *O*-chain of the external cell membrane. This is primarily confirmed by the fact that some RC-mutants are able to maintain phage T7 reproduction. Phage T7 particles are known to attach to the R-LPS of enterobacteria [10]. This phage is unable to adsorb on the cells of the parent strain ECA 62A and mutants with insignificant changes in the wild-type structure due to the absence of a direct physical contact with the receptor. On the other hand, S-LPS is the receptor for phage ZF40, which can attach only to the cells that have this lipopolysaccharide type. A similar situation was described earlier for the phage pair T7–P22 in a study of the interaction between coliphage T7 and *Salmonella typhimurium* cells [11]. As can be seen from Table 1 and Fig. 1b, the normal adsorption process for phage ZF40 is only possible in the presence of a certain intermediate type of S-LPS, which seems to be a constituent part of the cell membrane of mutants RC5297 and RC5501. Taking into account that, in mutant RC5297, the value of sensitivity to the lysing action of MCTV is changed insignificantly as compared to the parent strain (75%), my subsequent experiments with phage ZF40 and its different variants used this mutant as a universal indicator.

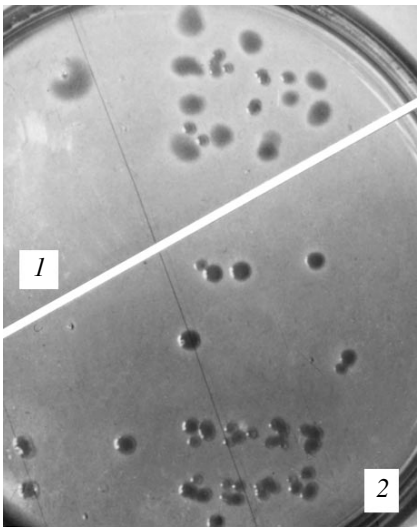

Fig. 2. Form of colonies of (*1*) the initial strain 62A of *E. carotovora* subsp. *carotovora* and (*2*) mutant RC5022 on LB plates.

A qualitative study of adsorption revealed that mutants RC5191, RC5192, and RC5195 indeed do not adsorb the bacteriophage ZF40 particles. Parallel experiments on the adsorption of MCTV particles established that all the resistant mutants (Table 1) did not possess the adsorption properties in relation to the selective MCTV and to the appropriate test carotovoricins. On the other hand, when grown on solid media, all the RC-mutants form a rough type of colonies, whereas the parent strain forms mucous colonies (Fig. 2). Thus, the S-LPS of *E. carotovora* 62A is the receptor not only

Table 2. Efficiency of inoculation of *E. carotovora* strains with different bacteriophage ZF40 variants

Strain	Group	Bacteriophage				
		ZF40/5297	ZF40/M2-4*	ZF40/33A*	ZF40/M2-4	ZF40/33A
62A	I	1	1×10^{-5}	3×10^{-5}	1	1
RC5297		0.9	2×10^{-6}	1×10^{-6}	1	1
M2-4		3×10^{-5}	1	1	4×10^{-5}	4×10^{-5}
33A	II	2×10^{-5}	0.7	1	4×10^{-5}	4×10^{-5}
37A		3×10^{-4}	–	–	–	–
61A	III	1×10^{-7}	1×10^{-4}	–	–	–
j15		1×10^{-8}	1×10^{-8}	–	–	–
15A		1×10^{-9}	1×10^{-8}	–	–	–
53A	IV	1×10^{-9}	1×10^{-9}	–	–	–
g125		1×10^{-9}	1×10^{-9}	–	–	–
j22		1×10^{-9}	1×10^{-9}	–	–	–
J2	V	0	1×10^{-7}	–	–	–
43A		0	1×10^{-9}	–	–	–
74A	VI	1×10^{-9}	0	–	–	–

for phage ZF40 but also for macromolecular carotovoricins.

When investigating the range of phage hosts among the *E. carotovora* strains, we used two phage ZF40 variants that were obtained on the initial strain ECA 62A and on the indicator RC5297 (phages ZF40 and ZF40/5297, respectively). Before determining the inoculation efficiency, the initial phage concentrations were no less than 1×10^{10} PFU/ml. As can be seen from Table 2, phage ZF40/5297 (ZF40) has a wide range of hosts among the ECA strains; two EAT strains are also sensitive to this phage.

Based on the inoculation efficiency of phage ZF40 as, well as of phage ZF40/M2-4* (see further), the *E. carotovora* strains maintaining the phage reproduction can be divided into six groups (Table 2). For two strains, ECA M2-4 and 33A, as well as for strain EAT 37A, the phage inoculation efficiency decreases to 10^{-4} to 10^{-5}, whereas the inoculation efficiency on the other strains is very low, and these strains produce a heterogeneous phage population, which is similar to that described above. In contrast to this, on strains ECA M2-4, ECA 33A, and EAT 37A, the phage forms equal-sized plaques after several passages (Fig. 1c).

To test the intracellular restriction in *E. carotovora*, Luria's classical test was used [12]. The subjects of the study were four ECA strains: 62A, RC5297, M2-4, and 33A. The phages obtained on M2-4 and 33A were cloned five times from separate colonies and concentrated as described above. The final titers of viable particles constituted no less than 1×10^{11} PFU/ml. The phages were designated as ZF40/M2-4* and ZF40/33A*, respectively. The restriction analysis of the DNA of these phages with endonuclease *Hpa*I showed that their genomes did not have any visible physical differences and were homologous to each other, as well as to the genome of the initial phage ZF40/5297–ZF40 (Fig. 3). The results obtained suggest that the restricted phage development in the cells of the ECA strains M2-4 and 33A was in fact connected with the occurrence of

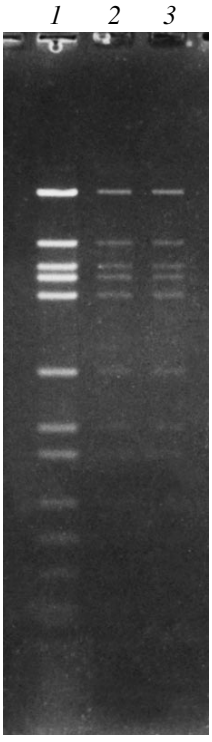

Fig. 3. Electrophoresis of the *Hpa*I-restriction DNA fragments of bacteriophages (*1*) ZF40/5297, (*2*) ZF40/M2-4*, and (*3*) ZF40/33A*.

Table 3. Characteristics of the artificial lysogens *E. carotovora* subsp. *carotovora* 62A (RC5297), M2-4, and 33A

Lysogen (phage)	Lysogen type	Rate of occurrence, %	Particle yield*		Superinfection result**
			A	B	
62A(ZF40)	I	100	+	2×10^5	4×10^9
RC5297(ZF40)	I	100	+	2×10^5	4×10^9
M2-4(ZF40*)	I	16	+	9×10^5	3×10^8
	II	16	–	4×10^2	–
	III	68	–	–	3×10^5
33A(ZF40*)	I	20	+	1.5×10^6	2×10^{10}
	II	40	–	–	–
	III	40	–	–	2×10^5

*The yield of particles was determined visually, (A) by the formation of lysis plaques or (B) by the phage particle titer after 24 h of lysogen growth.
**The superinfection result is expressed as the minimal amount of the viable phage particles forming a visible lysis plaque analogous to a MCTV plaque [5].

intracellular restriction. As in the other cases [12], the intracellular restriction in *E. carotovora* is closely coupled with the process of phage DNA modification. The latter is confirmed by the fact that phages ZF40/M2-4* and ZF40/33A* restore their initial inoculation efficiency on the appropriate strains (Table 2). To establish the presence of the restriction–modification system (the R–M system) in the initial strain ECA 62A and mutant RC5297, the lawns of these cultures were inoculated with the modified phages ZF40/M2-4* and ZF40/33A*. The primary titers of both phages decreased by an average of five orders; however, the initial IE was restored after four to five passages. Moreover, both phages restored the morphology and the size of the negative colonies on mutant RC5297 (Fig. 1b). In Table 2, these phage variants are designated as ZF40/M2-4 and ZF40/33A. Based on the quantitative data shown in this table, a conclusion about the complete identity of the R–M systems of strains M2-4 and 33A of *E. carotovora* subsp. *carotovora* can be made, whereas the restriction–modification system of strain ECA 62A, primarily restricting the reproduction of phages ZF40/M2-4* and ZF40/33A*, differs from the previous ones.

Analysis of the results obtained by studying the IE for phages ZF40/5597 and ZF40/M2-4* (Table 2) shows that the *E. carotovora* strains studied may have several different R–M systems. The possibility that unique restriction-modification systems are present in EAT 37A, ECA 61A, and ECA J2 cannot be ruled out. As to the other phage-sensitive erwinias, the restriction of phage ZF40 development by them is very significant. The inoculation efficiency of this bacteriophage and its modified variant on these strains is 1×10^{-9} to 1×10^{-8}, which may be related to the superimposition of two effects: hampered adsorption of phage particles on the cells and the action of intracellular restriction in the host bacterium.

As mentioned above, phage ZF40 forms negative colonies typical of temperate bacteriophages on mutant RC5297. In the case of a heterogeneous phage population or when the ECA strains M2-4 and 33A are infected, the secondary growth of the lysogens can be observed in confluent lysis (Figs. 1a, 2a, 3b). The characteristic plaque morphology is a stable phage trait and does not change with multiple bacteriophage passages on sensitive cells. That phage ZF40 is a temperate phage is also evidenced by the appearance of transparent negative colonies when the phage particles are produced by the confluent lysis method. The transparent plaques appear at a low rate and are to be assigned to the c-type of phage mutants. To definitively elucidate the nature of bacteriophage ZF40, I obtained ZF40-, ZF40/M2-4*-, and ZF40/33A*-based lysogens for the indicators 62A (RC5297), M2-4, and 33A, respectively. All the lysogens proved insensitive to the productive infection by these phages, and the phages themselves appeared, as should have been expected, to be homoimmune. The lysogens obtained for strain 62A (RC5297) were monotypic; when plated, they form negative zones on the lawn of nonlysogenized cells and have a level of spontaneous induction of 2×10^5 PFU/ml (Table 3). The lysogens of the modified variants ZF40/M2-4* and ZF40/33A* of the M2-4 and 33A cultures differ in the main parameters (Table 3). Their first type has features similar to those of strains ECA 62A (ZF40) and constitutes only 16 to 20% of the total amount of the lysogenic variants of strains M2-4 and 33A. The other two types of lysogens prevail quantitatively (about 80% for both strains) over the first type of lysogens. The latter lysogens are characterized either by an insignificant level of spontaneous induction or by its complete absence. My experiments showed that many natural *E. carotovora* strains manifest an unusual type of reaction to infection by phages ZF40 and ZF40/M2-4*: they form lysis plaques without phage reproduction. These lysis plaques are similar to those induced by macromolecular carotovoricins [5].

To explain this unusual phenomenon, all the lysogens were studied by the method of superinfection with a homologous bacteriophage. As a result of these investigations, it was established that type I lysogens behaved as classical lysogens and were insignifi-

cantly prone to the lysis induced by the homologous bacteriophage. In this case, in order to obtain a positive reaction, which is manifested in the formation of indistinct lysis plaques, large amounts of a superinfecting bacteriophage are necessary: from 3×10^8 to 2×10^{10} PFU/ml. For type 3 lysogens, this amount is less by several orders and constitutes, on average, 2×10^5 to 3×10^5 PFU/ml. Type 2 lysogens do not show any appreciable sensitivity to the superinfecting homologous bacteriophage (Table 3). A comparison of the infection of natural *E. carotovora* strains by phages ZF40 and ZF40/M2-4* and of superinfection of the artificial lysogens shows that these two processes have similar features. They reflect the resistance of phytopathogenic erwinias to bacteriophages at the level of the immune response of a lysogenic cell. This conclusion suggests that homoimmune temperate bacteriophages must be widely spread in the group of *E. carotovora* strains studied. To confirm this, concentrated lysates of strains ECA J2 and 55A obtained with the use of nalidixic acid (20 μg/ml, [5]) were tested for the presence of temperate bacteriophages using ECA 62A as an indicator. The usual erwinia lysates induced by mitomycin C [5] were tested using the universal indicator RC5297. As a result, five temperate phages were discovered: EF444, EF47, EF35, EF19, and EF36. These phages were unable to develop in the cells of lysogens shown in Table 3. None of the above-described bacteriophages—ZF40, ZF/M2-4*, and ZF40/33A*—was reproduced in the ECA 62A lysogenic cells carrying these temperate phages. As was to be expected, a larger number of temperate erwiniophages was revealed with the indicator RC5297 than with the initial sensitive strain ECA 62A.

Despite the fact that *E. carotovora* is an important phytopathogen, which is intensely studied in many laboratories, its bacteriophages do not number even a dozen [3, 4, 13]. This unique situation has no explanation so far. The erwiniophages are unlikely to possess special properties and to differ radically from other enterobacterial phages. Most probably, the cause of the rare occurrence of bacteriophages in *E. carotovora* should be sought at the level of the unusual phage resistance of the cells of this bacterium. In this work, I managed to show that the phage resistance of pectinolytic erwinia is determined by two main causes: hampered phage adsorption on the indicator cells and the presence of the host restriction systems. For temperate bacteriophages, phage resistance at the level of the immune response of lysogenic pectinolytic erwinias should also be taken into account. The rare discovery of temperate erwiniophages may also be due to the masking effect of bacteriocins of different nature, which does not allow viable temperate bacteriophages to be revealed upon lysogenic induction by appropriate agents; it may also be due to the presence of defective temperate bacteriophages forming macromolecular bacteriocins related to phage tails [5, 7].

REFERENCES

1. Chatterjje, A.K. and Starr, M.P., Genetics of *Erwinia* Species, *Annu. Rev. Microbiol.*, 1980, vol. 34, pp. 645–674.
2. Perombelon, M.C.M., The Genus Erwinia, *The Procaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, Balows, A., Ed., New York: Springer-Verlag, 1992, vol. 1, pp. 2899–2921.
3. Toth, I., Perombelon, M., and Salmond, G., Bacteriophage φKP Mediated Generalized Transduction in *Erwinia carotovora* subspecies *carotovora*, *J. Gen. Microbiol.*, 1993, vol. 139, no. 11, pp. 2705–2709.
4. Toth, I., Mulholland, V., and Cooper, V., Generalized Transduction in the Potato Blacked Pathogen *Erwinia carotovora* subsp. *atroseptica* by Bacteriophage φM1, *Microbiology*, 1997, vol. 143, no. 10, pp. 2433–2438.
5. Tovkach, F.I., Biological Properties and Classification of *Erwinia carotovora* Bacteriocins, *Mikrobiologiya*, 1998, vol. 67, no. 6, pp. 767–774.
6. Gorb, T.E. and Tovkach, F.I., Typing *Erwinia carotovora* Phytopathogenic Strains on the Basis of Pectinolytic Activity and Sensitivity to Bacteriocins (Carotovoricins), *Mikrobiologiya*, 1997, vol. 66, no. 6, pp. 823–828.
7. Tovkach, F.I., Relationship between the Lytic Activity of Macromolecular Carotovoricins and Bacteriocin Sensitivity of Producer Strains of *Erwinia carotovora*, *Mikrobiologiya*, 1998, vol. 67, no. 6, pp. 775–781.
8. Pagsly, E. and Oudega, B., Methods for Investigation of Colicin Plasmids, *Plazmidy: Metody* (Plasmids: Methods), Moscow: Mir, 1990.
9. Tovkach, F.I., Grigoryan, Yu.A., Ruban, V.I., Danileichenko, V.V., and Kishko, Ya.G., Restriction Map of Permutated DNA of the Temperate Bacteriophage 59 of *Erwinia carotovora*, *Mol. Genet., Mikrobiol. Virusol.*, 1988, no. 1, pp. 20–24.
10. Beumer, J., Hannecart-Pocorni, E., and Godard, C., Bacteriophage Receptors, *Bull. Inst. Pasteur*, 1984, vol. 82, no. 3, pp. 173–253.
11. Brunovskis, I. and Burns, R.D., Growth of Coliphage T7 in *Salmonella typhimurium*, *J. Virol.*, 1973, vol. 11, no. 5, pp. 621–629.
12. Kruger, D.H. and Bickle, T.A., Bacteriophage Survival: Multiple Mechanisms the Deoxyribonucleic Acid Restriction Systems of Their Hosts, *Microbiol. Rev.*, 1983, vol. 47, no. 3, pp. 345–360.
13. Tovkach, F.I., Grigoryan, Yu.A., Ruban, V.I., and Kishko, Ya.G., Polylysogeny in *Erwinia carotovora* 268 R, *Mikrobiol. Zh.*, 1984, vol. 46, pp. 71–78.