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EXPERIMENTAL ARTICLES

The Study of *Erwinia carotovora* Bacteriocins with the Aid of Nalidixic Acid–Resistant Bacterial Indicator Cells

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Abstract—A novel approach is proposed for the study of the macromolecular bacteriocins of *Erwinia carotovora* (MCTVs). The approach lies in the bacteriocinogeny of pectolytic erwinia being studied using a lawn of a bacterial mutant resistant to nalidixic acid, an inducer of MCTVs. The high efficiency of this approach was demonstrated by studying carotovoricins in 104 different *E. carotovora* strains, 88% of which bear MCTVs, distinguished by the morphology of zones of induced lysis on a lawn of susceptible cells, the lysis pattern, and some other characteristics. Preliminary studies using this approach showed that there is no correlation between the occurrence of MCTVs in particular *E. carotovora* strains and the habitat of the host plants from which these strains were isolated. There are grounds to believe that the approach proposed can also be used for investigating bacterial lysogeny.

Key words: *Erwinia carotovora*, mutants, bacteriocins, properties, ecology.

Erwinia carotovora strains produce two types of bacteriocins, colicin-like carotovoricins (CCTVs) and phage-tail-like macromolecular carotovoricins (MCTVs) [1–3]. The structure of MCTVs [4, 5], the nature of their cell receptors [6], the mechanism of their action on cells [7], and their genetic determinants [8–10] have been fairly well studied. MCTVs were successfully used for typing phytopathogenic *E. carotovora* strains [11]. MCTVs are widely spread in *E. carotovora* strains but not in other enterobacteria [1–3, 12], which implies that MCTVs play an important part in the ecology of erwinia and may serve as a species-specific indicator of phytopathogenic pectolytic erwinia [3, 12].

In this work, we attempted to develop a simple approach for the detection and investigation of macromolecular carotovoricins.

MATERIALS AND METHODS

Experiments were carried out with 48 strains of *Erwinia carotovora* subsp. *carotovora* (ECA strains), *Erwinia carotovora* subsp. *atroseptica* (EAT strains), and *Erwinia aroideae* (EAR strains), which were listed in previous publications [3, 11]. The other 56 strains studied in this work are described in Table 1. Forty-six of these strains were obtained from the collections of microorganisms at the Department of Phytopathogenic Bacteria (head R.I. Gvozdyak), Zabolotnyi Institute of Microbiology and Virology, and the Department of Microbiology (head Yu.K. Fomichev), Belorusian State University. The remaining ten strains were isolated by F.I. Tovkach.

Macromolecular carotovoricins were studied as described earlier [3, 12]. *E. carotovora* mutants resistant to nalidixic acid (Nal^r mutants) were obtained as described in the handbook [13]. The bacteriocinogeny of pectolytic erwinia was studied by the agar layers method, which is usually used for the titration of bacteriophages [13]. The lower layer was 1.2% LB agar, and the upper layer was 0.5% soft agar containing 20 μg/ml nalidixic acid and Nal^r indicator cells. Experiments with such agar plates (called NAIC plates) were carried out as follows. Nalidixic acid and the suspension of Nal^r indicator cells were added to molten soft agar occurring at a temperature of 45–50°C. After thorough mixing, the molten agar was pour-plated onto the surface of 1.2% LB agar. Test erwinia cells, which were preliminarily grown for 2–3 days on conventional 1.2% LB agar plates, were transferred to NAIC plates by means of replica plating. Alternatively, liquid suspensions containing from 5×10^8 to 4×10^9 test cells/ml or their tenfold dilutions were applied, in 3- to 5-μl drops, directly onto the surface of NAIC plates. After the drops had dried, the plates were inverted and incubated at 25°C for 18–24 h. The control plates (called IC plates) contained only indicator cells without nalidixic acid. To study the induction of carotovoricins by pectin, we used NAIC plates whose lower layer was agarose gel with pectin. This medium was prepared by mixing equal volumes of 2% agarose gel and 2% pectin solution with a 1/10 volume of 20× minimal A medium [13].

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Table 1. The *E. carotovora* strains studied in this work

Strains	Author	Original name/host plant/year of deposition in the IMV collection
144a	S.S. Sidorenko	*E. aroideae*[1]/*Brassica oleracea*/1956
482 E, 915, 741, 808a, 184	L.V. Kabashna	*E. aroideae*[1]/*Calla aethiopica* and *Hyacinthus orientalis*
47a , K–47	A.P. Korobko	*E.toxica*[1]/*Cucumis sativus*/1967 and 1970
7869*	–	*E. aroideae*[1]/*Lycopersicum esculentum*/1956
B2, B3, B16	F.I. Tovkach	*Erwinia* sp.[1]/*Solanum tuberosum*/2001
B4, B10, B11, B12, B13, B14, B15	F.I. Tovkach	*Erwinia* sp.[1]/*Daucus sativus*/2001
24A, 40A, 71A, j15	A.N. Evtushenkov	*E. carotovora* subsp. *carotovora*[2]/S. *tuberosum, Iris* sp.
4(2), M2–8, 45–2, 48–1, 59–2, 78–3, 167–1	A.N. Evtushenkov	*Erwinia* sp.[2]/*S. tuberosum*/1987
48 P, 53 P, 91 P	L.K. Pavlova-Ivanova	*E. carotovora*[3]/*Iris* sp., *Hyacinthus orientalis*/1975 and 1976
2054	Yu.I. Shneider	*E. carotovora*[4]/*S. tuberosum*/ 1967
9 F	Yu.I. Shneider	*E. atroseptica*[4]/*S. tuberosum*/1967
133, 180, 495	–	*E. carotovora*[5]/*B. oleracea* and *D.sativus*
718	–	*E. aroideae*[5]/*D. sativus*
246	–	*E. aroideae*[6]/*B. oleracea*
258	–	*E. carotovora*[6]/*B.oleracea*
2	E.V. Matveeva	*E. carotovora*[7]/*Helianthus annuus*/1995
921	R.M. Galach'yan	*E. carotovora*[8]/*D. sativus*/1959
NCPPB 550	–	*E. aroideae*[9]/*Nicotiana tabacum*
SR 1	–	*E. atroseptica*[9]/*S. tuberosum*
EG 48 (=G 148 = ATCC 27388 = 8510* = UKM B-1148 = NCPPB 1065	K.A. Sabet (Egypt, 1961)	*E. carotovora f. zeae*[9]/*Zea mays*/1970
G 147	–	*E. carotovora f. zeae*[9]/*Z. mays*
G 117	–	*E. carotovora*[9]
CCM 1008^T = ATCC 15713^T = NCPPB 312^T	E. Hellmers (Denmark)	*E. carotovora*[10]/*S. tuberosum*
CCM 1011 = NCPPB 438	–	*E. carotovora*[10]/*Iris* sp.
SR 165	A. Kelman (Minnesota)	*E. carotovora*[11]/*S. tuberosum*
E 193	R.J. Copeman (British Columbia)	*E. carotovora*[11]/*S. tuberosum*
162	H. Maas-Geesteranus (The Netherlands)	*E. carotovora*[11]/*S. tuberosum*
209	S.H. De Boer (British Columbia)	*E. carotovora*[11]/*S. tuberosum*
Cc110	M. Powelson (Oregon)	*E. carotovora*[11]/*S. tuberosum*
NCPPB 549^T = LMG 2386^T = ATCC 33260^T	D.C. Graham (United Kingdom)	*E. atroseptica*[12]/*S. tuberosum*

* Strain code in the IMV (Institute of Microbiology and Virology) collection. ** The year of deposition in the IMV collection is unknown. "–" stands for "no data available". UKM, Ukrainian Collection of Microorganisms; ATCC, American Type Culture Collection; NCPPB, National Collection of Plant Pathogenic Bacteria, United Kingdom; CCM, Czech Collection of Microorganisms. 1, IMV, Ukraine; 2, Department of Microbiology, Belarusian State University, Belarus; 3, Botanical Garden, Moscow, Russia; 4, Research Institute of Potato Growing, Russia; 5, Research Institute of Agricultural Microbiology, Russia; 6, Research Institute of Phytopathology, Russia; 7, F.S. Dzhalilov, Timiryazev Agricultural Academy, Russia; 8, Institute of Microbiology, Armenia; 9, A. Lazar, Institute of Biology, Romania; 10, M. Kocur, Czech Collection of Microorganisms; 11, S.H. De Boer, Canada; 12, J. De Ley, Belgium.

RESULTS AND DISCUSSION

Many convenient methods are presently available that can detect small proteinous colicin-like bacteriocins [14]. However, most of these methods are inappropriate for the study of other bacteriocins, such as phage-tail-like macromolecular bacteriocins, whose diffusion in agar media is very low [2, 3]. In this work, we attempted to apply a basically novel approach to the

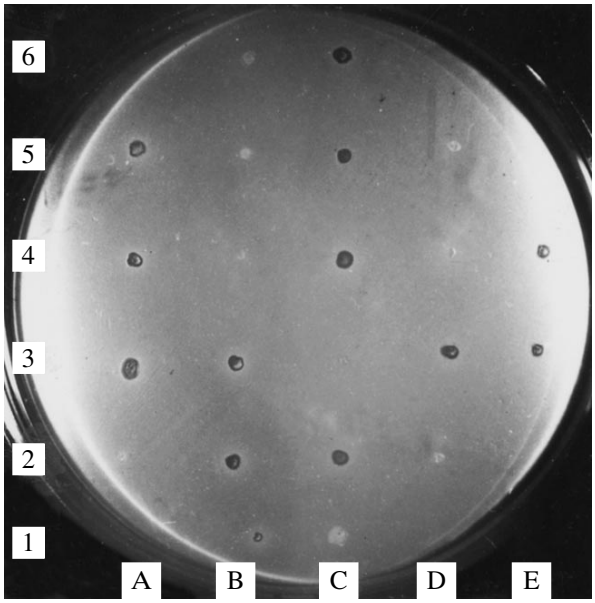

Fig. 1. The induction of MCTVs on a lawn of the Nal^r ECA 62A (RN50) indicator strain. Figures at the left and capital letters at the bottom define the coordinates of bacterial colonies transferred onto the lawn by the method of replica plating: (B1) ECA SR165; (C1) EAT g125; (A2, D2) EAT Mg147/43; (B2) ECA 59A; (C2) ECA 69A; (A3) ECA 74A; (B3) ECA M2-4; (C3) EAR 566VKM; (E3) ECA 43A; (B4) ECA C366; (C3) ECA 54A; (D4) ESP ZM-1; (E4) ECA 51A; (A5) EAT 52A; (B5) ESP B1; (C5) ECA 4A; (D5) ECA 52A; (B6) EAT 39A; (C6) ECA 50A. ESP stands for *Erwinia* sp.

investigation of macromolecular carotovoricins. The approach lies in the creation of optimal conditions for the induction of MCTVs (for this purpose, we used nalidixic acid) and their detection with the aid of a lawn of nalidixic acid–resistant (Nal^r) indicator cells susceptible to carotovoricins. If nalidixic acid–sensitive erwinia cells are applied on such a lawn grown on an agar layer containing 20 μg/ml nalidixic acid, the cells will lyse, releasing biologically active carotovoricins. If the Nal^r indicator cells are susceptible to these carotovoricins, clear zones of lysed indicator cells will appear on the lawn.

Earlier, two large bacteriocinotypes (BT1 and BT2) were revealed among *E. carotovora* strains, the strains of bacteriocinotype BT1 being slightly susceptible to MCTVs and the strains of bacteriocinotype BT2 being highly susceptible to MCTVs [12]. To study the bacteriocinogeny of 104 phytopathogenic pectolytic *E. carotovora* strains, we used seven indicator strains of bacteriocinotype BT2 highly susceptible to carotovoricins (the ECA strains 54A, M2, M3, M4, 66A, and 59A and the EAT strain g217) and three strains of bacteriocinotype B1 characterized by low susceptibility to MCTVs (the ECA strains 52A and 35A and the EAR strain 3A). From all of the indicator strains, we derived nalidixic acid–resistant mutants (called RN mutants), which could grow in the presence of nalidixic acid at concentrations of no less than 20 μg/ml. As was expected, the plating of some tested *E. carotovora* strains onto a lawn of Nal^r indicator cells caused the formation of zones of lysis on the lawn due to the killer activity of carotovoricins [3]. The plating of the same strains onto the control IC plates containing no nalidixic acid, as a rule, did not give rise to zones of lysis on the lawn. Figure 1 exemplifies the testing of 19 different MCTV-bearing erwinia strains on a plate with a lawn of the Nal^r indicator cells of strain RN50, which was derived from the ECA strain 62A.

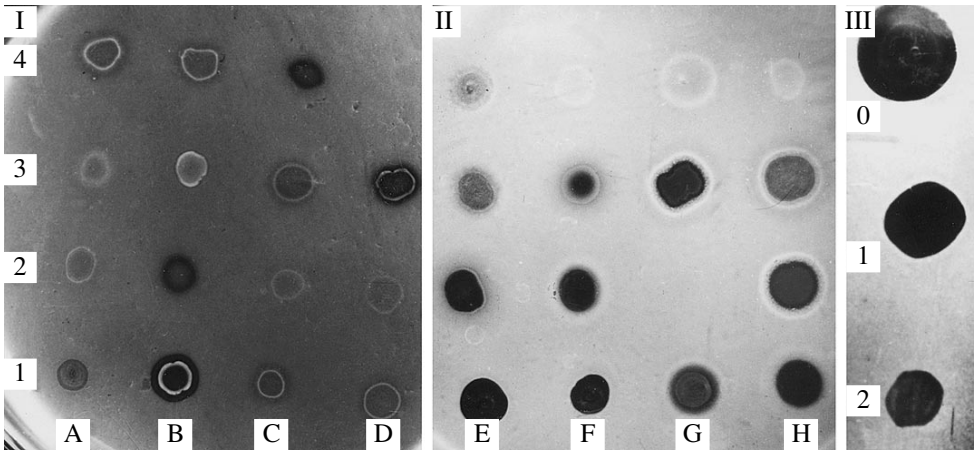

Fig. 2. The morphology of zones of lysis induced by carotovoricins on a lawn of the Nal^r ECA 66A (RN183) indicator strain. Plate I: suspensions of stationary-phase cells ($2–4 \times 10^9$ cells/ml) were applied onto the lawn in 3-μl aliquots. Plate II: fresh suspensions ($5–7 \times 10^8$ cells/ml) were applied onto the lawn in 3-μl aliquots. Plate III: zones of lysis induced by the MCTVs of ECA 53A (figures 0, 1, and 2 mark, respectively, undiluted, diluted tenfold, and diluted hundredfold cell suspensions). (A1, H4) EAT 39A; (B1, G1) ECA J2; (C1) ECA 2054; (D1, E1, F1) EAR NCPPB550; (A2, H2) ECA Ec153; (B2, H1) ESP ZM-1; (C2) ESP 59-2; (D2) ETO K-47; (E2, F2) ECA SR165; F2, B4) ECA 69A; (G2) ECA 921; (A3, D4, G3) EAR 3A; (B3, F4) EAR 4A; (C3) ESP 45-2; (D3, H3, A4) EAR g48; (E3) EAR 566VKM; (B4, G4) ECA 52A; (E4) ECA 15A. ETO stands for *Erwinia toxica.*

Table 2. The efficiency of detection (expressed as a percent) of macromolecular carotovoricins in various *E. carotovora* strains

Indicator			Inducer		
strain	bacteriocinotype	Nal^r mutant	mitomycin C	NA	NA
ECA 54A	2	RN416	60	46	51
ECA M2-4	2	RN507	60	56	49
ECA 66A	2	RN183	73	54	47
ECA 59A	2	RN558	46	33	47
ECA 62A	2	RN50	58	52	44
ECA 50A	2	RN324	48	21	24
ECA 52A	1	RN355	10	13	15
EAR 3A	1	RN112	17	17	13
ECA 35A	1	RN122	8	6	7
EAT g217	2	RN579	40	0	2

Note: The efficiencies of MCTV detection presented in columns 4 and 5 were determined from data for the same 48 *E. carotovora* strains. The efficiencies of MCTV detection presented in column 6 were determined from data for all 104 *E. carotovora* strains studied.

Table 3. The distribution of the MCTVs of different *E. carotovora* strains over different groups of lysis as determined with six Nal^r indicator strains

Parameter	Country			
	Belarus	Ukraine	Russia	other countries*
Number of strains	48	21	14	21
Number of lytic groups	21	12	9	12
Frequency of groups	0.44	0.57	0.71	0.62
Distribution frequency over groups:**				
1, 2	0.44	0.42	0.43	0.38
4, 5	0.08	0.10	0.14	0.14
9–11	0.08	0.05	0.21	0.10

* Data in this column refer to *E. carotovora* strains isolated in Armenia, Egypt, Europe, the United States, and Canada (the collections of microorganisms designated in the Notes to Table 1 by figures 8 through 12).

** These groups include different numbers of MCTVs.

The evaluation of the efficiency of carotovoricin detection in various erwinia strains as a percent of sensitivity [3] showed that the new approach is a reliable method for the study of bacteriocinogeny (Table 2). The results obtained by this method confirmed the earlier observation that mitomycin C is a more efficient inducer of bacteriocins than nalidixic acid [3]. The efficiency of MCTV detection with the use of the Nal^r indicator strains of bacteriocinotype BT2 was comparable with that attainable with mitomycin C. As for the three indicator strains of carotovoricinotype BT1, they showed either the same or even higher efficiency than that provided by the conventional method [3]. The only exception was the RN579 mutant of *E. carotovora* subsp. *atroseptica* strain g217, which exhibited a very low efficiency of MCTV detection with nalidixic acid (2%), while the efficiency of MCTV detection with mitomycin C was as high as 40% (Table 2).

Various Nal^r indicator strains showed an efficiency of MCTV detection ranging from 7 to 51% (Table 2). It should be noted that most carotovoricins are inducible, so that the percent of the spontaneous release of MCTVs on the control plates without nalidixic acid was low (from 0 to 2%). The substitution of LB medium by AP medium in NAIC plates did not markedly affect the efficiency of MCTV detection.

The new approach showed that 91 of the 104 erwinia strains tested (i.e., about 88%) contain biologically active MCTVs. Such carotovoricins were not detected in only seven ECA strains, five EAT strains, and one EAR strain. However, the electron microscopic examination of the mitomycin C–induced lysates of some of these strains showed that they actually contain phage-tail-like MCTVs [12]. Presumably, carotovoricins of this kind can be detected with indicator strains other than those used in this work.

The fact that the bacteriocinogeny of erwinia strains depends neither on the geographic location of their host plants, the year of isolation, nor on the primary species name (Table 1) suggests that the genetic determinants of MCTVs are conservative. This conservatism and the wide distribution of MCTVs among erwinia strains confirm the earlier suggestion [3, 12] that the content of MCTVs may be considered as a species-dependent characteristic of *E. carotovora*.

Further studies showed that RN mutants can be used for the study of such biological properties of MCTVs that cannot be investigated using the mitomycin C–induced lysates of erwinia cells.

To study the characteristics of zones of lysis on a lawn of indicator cells that can be considered as inherent characteristics of particular carotovoricins, we used a variant of the approach in which liquid suspensions of test strains were applied, in 3- to 5-μl drops, directly onto the surface of NAIC plates. This allowed us to establish that the age of erwinia cultures considerably influences the efficiency of carotovoricin induction and the morphology of zones of lysis on a lawn of indicator cells. Specifically, the use of stationary-phase cells resulted in a low efficiency of MCTV induction on NAIC plates. In this case, zones of lysis on the lawn often contained circular regions in which cells were not lysed (Fig. 2, plate I, zones A4, B1, C1, D1, and D2). Some strains gave rise to zones of lysis typical of those produced by CCTVs (Fig. 2, plate I, zones A4, B4, C1, and D1). At the same time, logarithmic-phase erwinia cells applied onto a lawn of indicator cells gave rise to

Table 4. The geographic distribution of *E. carotovora* strains bearing MCTVs of different groups

MCTV group	Lysis					Total number of strains with particular MCTVs	Countries	Number of host plants
	RN mutant							
	416	507	183	558	50			
II	+	+	+	+	–	10	Belarus (4), Ukraine (3), Russia (2), Armenia (1)	4
III	+	+	+	–	+	10	Belarus (10)	2
IV	+	+	–	+	+	4	Russia (2), Belarus (1), other countries (1)	2
VI	+	+	+	–	–	11	Ukraine (9), Belarus (2)	4
X	+	–	+	+	–	4	Ukraine (2), Belarus (2), United States (1)	3
XXI	–	–	–	+	+	6	Belarus (5), other countries (1)	2
XXV	–	–	–	–	+	12	Belarus (7), Ukraine (1), Russia (2), other countries (2)	4

Note: The symbols "+" and "–" indicate the presence or absence of lysis, respectively. In parentheses are the number of *E. carotovora* strains bearing the MCTVs of particular groups.

distinct zones of lysis, which can be considered as inherent characteristics of particular MCTVs (Fig. 2, plate II). Some of these zones contained peripheral regions, which differed in their shape, size, and density.

An analysis of the dependence of the size of zones of lysis on the concentration of cells applied onto a lawn of Nal^r indicator cells showed that there are two kinds of MCTVs. The MCTVs of the first kind gave rise to clear zones of lysis, whose size decreased with decreasing the concentration of the applied cell suspensions (Fig. 2, plate III), whereas the MCTVs of the second kind gave rise to translucent zones of lysis of an equal size, whose turbidity increased with decreasing the concentration of the applied cell suspensions. Different MCTVs produced visible zones of lysis at different cell concentrations, from 2×10^3 to 2×10^6 cells per 5-μl drop. By this property, MCTVs can be divided into at least three groups, as is evident from the results of examination of five test strains with two indicator mutants, RN50 and RN183.

MCTVs also differed in the pattern of the induced lysis of six Nal^r indicator strains (RN416, RN507, RN183, RN558, RN50, and RN324) (Table 2). Five of these indicator strains (except for RN324) were used, either individually or in different combinations of four strains, for the determination of the lytic characteristics of MCTVs [3, 12]. Analysis of the frequency characteristics of MCTVs for four indicator strains showed that, according to their lytic characteristics, all 91 MCTVs can be divided into 16 groups. For five indicator strains, we observed 25 variants of lysis (of 32 possible). For six indicator strains, the frequency of MCTV distribution over different lytic groups was 0.58 (37 lytic variants of 64 possible). In all three cases, particular groups of lysis included from 1 to 12 similar MCTVs. The number of lytic groups consisting of one MCTV typically increased as the number of indicator strains increased.

The availability of a large number of carotovoricin-bearing *E. carotovora* strains isolated from different sources made it possible to investigate their ecological characteristics. Table 3 summarizes the frequency characteristics of different lytic groups of MCTVs from erwinia strains isolated in different countries (Belarus, Ukraine, Russia, and other countries). Analysis of these characteristics showed that the distribution of carotovoricins over different groups of lysis does not depend on the geographic location of the site of isolation. The frequencies of the lytic groups of nearly all MCTVs (except for those borne by Belarusian erwinia strains) differ insignificantly. A slightly higher frequency of the lytic groups of MCTVs of Russian strains (0.71) as compared with that of MCTVs of Ukrainian strains (0.57) and erwinia strains from other countries (0.62) may result from the relatively small number of Russian strains (14 in comparison with 21 isolated in Ukraine and in other countries). Furthermore, we found no correlation between the lytic properties of MCTVs and the site of isolation of the MCTV-bearing strains when three countries (Russia, Ukraine, and Belarus) were considered as one geographic region.

A comprehensive analysis of the lytic groups of carotovoricins with allowance for the number of MCTVs in particular groups (the groups were formed for five indicator strains) showed that some groups (groups II, IV, and VI in Table 4) include the MCTVs of erwinia strains isolated in neighboring countries. One of the most abundant groups (group III) included carotovoricins typical of a narrow ecological niche, namely, the MCTVs of Belarusian erwinia strains. These MCTVs turned out to be entirely identical to those obtained with mitomycin C and analyzed using 52 indicator strains (group IV) [3, 12]. At the same time, the erwinia strains bearing these carotovoricins differed in pectolytic activity [11], antibiotic resistance, the number of plasmids [15], and in some other characteristics. This suggests that the genetic determinants of MCTVs are relatively autonomous and might have been

acquired by particular strains through their horizontal transfer. On the other hand, the possibility cannot be excluded that the genetic determinants of MCTVs remain conservative when *E. carotovora* strains undergo certain changes in the course of their adaptation to a varying environment.

As can be seen from Table 4, some MCTVs characterized by the same type of lysis of susceptible indicator cells are borne by erwinia strains isolated from different sources (Table 1). This fact provides further evidence for the absence of correlation between the bacteriocinogeny of a strain and the source from which this strain was isolated.

Thus, the bacteriocinogeny of *E. carotovora* strains can be studied by a simple and informative method which is based on the use of nalidixic acid–resistant indicator cells. This method can be employed not only for the identification of carotovoricins and the study of their biological properties, but also for the study of carotovoricin ecology. The high efficiency of this method was confirmed in experiments with erwinia mutants with an enhanced and decreased ability to synthesize carotovoricins (F.I. Tovkach, unpublished data). Furthermore, preliminary experiments showed that this method can be used for the study of not only carotovoricins but also the bacteriocins of other phytopathogens and epiphytes (such as *Pseudomonas* spp. and *Erwinia herbicola*), as well as for the study of bacterial lysogeny.

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