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EXPERIMENTAL ARTICLES

Isolation and Preliminary Characterization of Cryptic Plasmids from *Erwinia carotovora*

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Abstract—Of the fifty-two *Erwinia carotovora* strains studied, sixteen were found to contain extrachromosomal DNA (plasmids) from 2.5 to 129 kbp in size. Some *E. carotovora* strains bore two to five different plasmids. Experiments showed that the cryptic plasmids of erwinia are not responsible for their resistance to antibiotics and are not involved in the synthesis of macromolecular colicin-like carotovoricins. At the same time, one of the *E. carotovora* strains, 13A, augmented the production of carotovoricin after curing from one of its plasmids, the 47.7-kbp pCA 6-2. Three *E. carotovora* subsp. *carotovora* strains and one *E. carotovora* subsp. *atroseptica* strain contained large 129-kbp plasmids, which may play a role in the ecology of phytopathogenic pectinolytic erwinia.

Key words: *Erwinia carotovora*, plasmids, isolation, DNA size.

Enterobacterial plasmids, including those of the *Erwinia* species (*E. amylovora, E. herbicola*, and *E. uredovora*), have been extensively studied [1, 2, 6–8]. At the same time, little is known about the plasmids of another *Erwinia* species, *E. carotovora* [2–5].

The aim of the present work was to study the cryptic plasmids of the phytopathogenic *E. carotovora* strains that were characterized earlier with respect to pectinolytic activity, production of bacteriocins, and sensitivity to these killer proteins.

MATERIALS AND METHODS

Two *Erwinia* sp. strains, B1 and ZM-1 [9]; thirty-seven *Erwinia carotovora* subsp. *carotovora* strains, Ec153 (ATCC 15359 = NCPPB 1847), J2 (NCPPB 1744), g179, C366, g123, B566 (VKM B-566), 216, j13, j22, 4A, 7A, 13A, 15A, 19A, 31A, 33A, 35A, 42A through 45A, 48A, 50A through 52A, 54A, 55A, 58A, 59A, 61A, 62A, 64A, 66A, 69A, 70A, 74A, and M2-4; seven *Erwinia carotovora* subsp. *atroseptica* strains, 36, Mg147/43, g217, g125, 36A, 37A, and 39A; and six *Erwinia aroidea* strains, 3A, 24A, 28A, 9BII, g10, and g48, were obtained from Yu.K. Fomichev, the Department of Microbiology of the Belarusian State University. The strains differed in the origin, the range of host plants, phytopathogenicity, pectinolytic activity, and sensitivity to macromolecular and colicin-like carotovoricins [9–11]. The *Agrobacterium tumefaciens* strain C58, which carries plasmid pTi-C58 [12], was obtained from S.E. Rymar', Institute of Molecular Biology and Genetics (IMBG) of the National Academy of Sciences of Ukraine. The *Escherichia coli* strains J53 and J63, which carry plasmids RP4 and RSF1010, respectively, were obtained from N.A. Kozyrovskaya, IMBG. Two variants of *E. coli* AB257(Hfr3000) and phage MS2 were obtained from T.P. Pererva, IMBG. One of these variants was found to contain about 2% of cells that were resistant to lysis by phage MS2. This spontaneous phage-resistant segregant (designated AB257-2) with an unintegrated sex factor was used as the source of plasmid F.

To isolate plasmids, *Erwinia* cells were grown in LB broth or in a minimal essential A medium [13] supplemented with 1% pectin or 10% potato slices as the source of protopectin. The growth temperature for *E. carotovora* and *A. tumefaciens* cultivated in LB broth was 25°C. Erwinia were cultivated for 1–2 days under intense aeration. The *E. coli* strains J53 and J63 were grown in LB broth at 30°C for 1 day. Cells for inoculation were preliminarily plated on LB agar containing either 50 μg/ml kanamycin or 15 μg/ml streptomycin (for J53 and J63, respectively).

Plasmid DNA was isolated using standard procedures [14, 15]. *E. carotovora* plasmids were detected and plasmids RSF1010, RP4, F, and pTi-C58 were isolated using the method of Kado and Liu [5]. Cells precipitated by centrifugation from a 1.5-ml aliquot of a 1- to 2-day-old bacterial culture were resuspended in 100 μl of 40 mM Tris–acetate buffer containing 2 mM Na_2EDTA (pH 7.9) and the cell suspension was mixed with two volumes of a lysing solution containing 3% SDS in a 50 mM Tris–HCl buffer (pH 12.6). The mixture was incubated at 55°C for 30 to 60 min and treated

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with a phenol–chloroform (1 : 1, v/v) solution. After centrifuging the resultant mixture at 11000 *g* for 10 min, the aqueous phase was carefully sucked using a plastic wide-opening pipette. An aliquot of this aqueous phase 20 to 40 μl in volume was analyzed for the presence of plasmids by electrophoresis in 0.7–1% agarose gel with buffer E [5].

The rest of the aqueous phase, about 200 μl in volume, was mixed with 1/10 volume of 3 M sodium acetate and then with two volumes of 96% cold ethanol (–20°C). The resultant precipitate, which represented plasmid DNA with trace amounts of RNA and chromosomal DNA, was washed four times with 80% ethanol, dried at room temperature, and dissolved in 50 μl of deionized water. The reaction mixture for restriction analysis contained 5 μl of a sample, 2 μl of tenfold-strength restriction buffer, 1–2 μl of an endonuclease (up to 4 units), and 11–12 μl of H_2O. Following incubation at 37°C for 2 h, restriction fragments were separated by electrophoresis in 0.9 or 1% agarose gel as described earlier [16]. The nucleases used in this work were *Bam*HI, *Eco*RI, *Eco*RII(*Mva*I), *Eco*RV, *Hind*III, *Hpa*I, *Pst*I, *Pvu*II(*Cfr*6I), and *Sal*GI purchased from Fermentas (Lithuania), Biopor (Russia), and Biolab (Russia). Endonucleases *Cfr*6I and *Mva*I were a gift from A.A. Yanulaitis (Vilnius, Lithuania).

The molecular mass of plasmids was determined by summing the molecular masses of their restriction fragments or by electrophoresis with the known plasmids—pTi-C58 (119 MDa), F (63 MDa), RP4 (36 MDa), RSF1010 (5.7 MDa), and pUC18 (1.75 MDa) [1, 12].

The antibiotic resistance of erwinia was determined using commercial paper disks impregnated with antibiotics (10 or 30 μg per disk). When it was necessary to use other antibiotic concentrations (5, 15, and 25 μg/disk), we used home-made antibiotic-impregnated paper disks. The range of tested antibiotics included ampicillin, carbenicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, penicillin, methicillin, monomycin, neomycin, oleandomycin, oxacillin, polymyxin, ristomycin, streptomycin, and tetracycline.

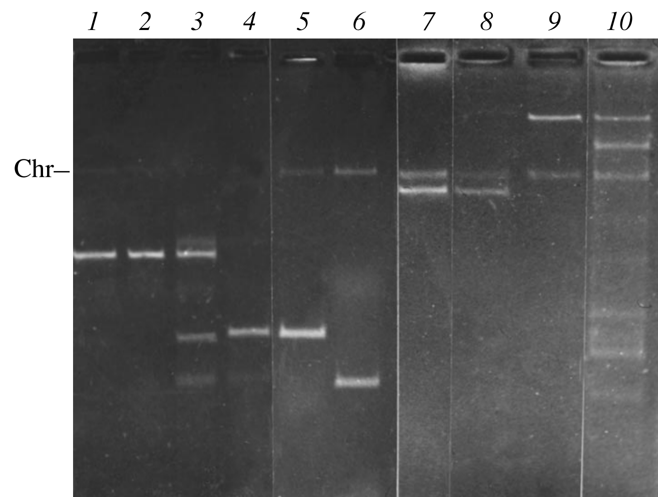

Fig. 1. Electrophoresis in 1% agarose of the plasmid DNA of strains (*1*) B556, (*2*) 48A, (*3*) 55A, (*4*) M2-4, (*6*) 52A, and (*10*) 13A of *E. carotovora* subsp. *carotovora* and strains (*5*) 36, (*7*) g152, (*8*) 39A, and (*9*) g217 of *E. carotovora* subsp. *atroseptica.* Chr, remnant bacterial chromosome. To isolate plasmids, *E. carotovora* cells were grown in LB broth for 1 day.

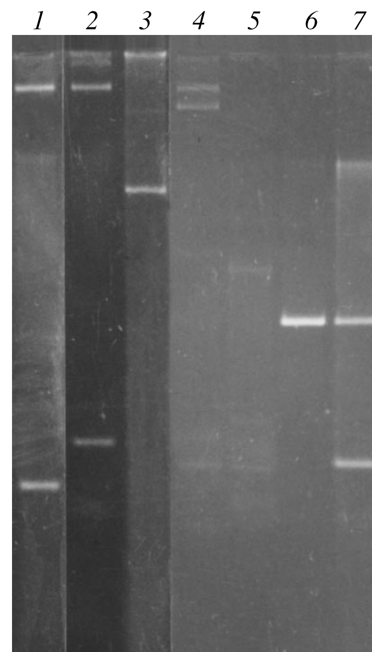

Fig. 2. Electrophoresis in 0.9% agarose of the plasmid DNA of strains (*1*) C366, (*2*) 33A, (*4*) 13A, (*5*) 35A, (*6*) 48A, and (*7*) 55A of *E. carotovora* subsp. *carotovora* and (*3*) *E. carotovora* subsp. *atroseptica* strain 39A grown in minimal essential A medium with 1% pectin for 48 h.

RESULTS AND DISCUSSION

The isolation of plasmids from phytopathogenic bacteria, including *E. carotovora* strains, is difficult because of the specific structure of their cell walls and the presence of interfering cellular components [5]. The failure of the earlier attempts to detect extrachromosomal DNA in bacteria of the genus *Erwinia* may be related to the use of inappropriate methods for the isolation of plasmids [3, 4].

The attempts to isolate plasmid DNA from erwinia cells by the methods commonly used for *E. carotovora* [14, 15] were not successful. Treatment with the nonionic detergents Triton X-100 (2.5%) and Brij 58 (2.5%) in combination with pronase B (50–100 μg/ml) at 60°C for 10–20 min made it possible to isolate only small plasmids with molecular masses from 1.6 to 6.0 MDa. Another disadvantage was a low reproducibility of the isolation method used. At the same time, the method of Kado and Liu [5], in which bacterial cells

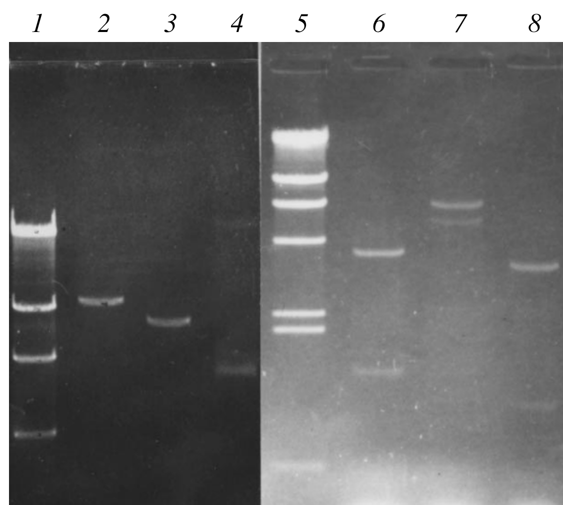

Fig. 3. Electrophoresis of plasmid DNA before and after digestion with restriction endonucleases. Lanes: *1* and *5*, the *Hin*dIII digest of DNA from phage lambda; *4*, plasmid pCA25 from strain 48A; *2* and *3*, plasmid pCA25 digested with *Pvu*II and *Hpa*I, respectively; *6*, *7*, and *8*, plasmid pAT52 from strain 36 digested with *Hpa*I, *Pvu*II, and *Eco*RV, respectively.

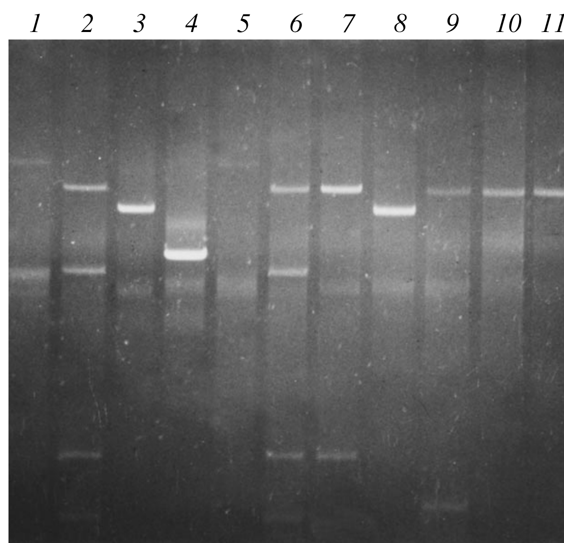

Fig. 4. Electrophoresis in 0.7% agarose gel of the cryptic plasmids of *E. carotovora* and reference plasmids from *A. tumefaciens* (pTi-C58), *E. coli* AB257-2 (plasmid F), and *E. coli* J53 (plasmid RP4). Lanes: *1* and *4*, plasmid pTi-C58; *3* and *8*, plasmid F; *4*, plasmid RP4; *2* and *6*, plasmid from strain 13A; *7*, plasmid from strain 13A-6/15; *9*, plasmid from strain 33A; *10*, plasmid from strain C366; and *11*, plasmid from strain g217.

are treated with 1.5% SDS and then chromosomal DNA is irreversibly denatured, whereas circular plasmid DNA is renatured, was found to be appropriate to the isolation of plasmids not only from *E. coli* and *A. tumefaciens* cells but also from *E. carotovora* cells (Fig. 1).

The investigation of the effect of nutrient media on the yield of plasmid DNA showed that it increased when strains 33A, C366, 48A, 55A, and 39A were grown in A medium with 1% pectin (Fig. 2) or 10% potato slices (data not presented) for 48 h. In this case, plasmid DNA was almost free of chromosomal DNA impurities (Fig. 2), as well as of RNA and proteins. These preparations of plasmid DNA were used for restriction analysis carried out with the restriction enzymes *Bam*HI, *Eco*RI, *Eco*RII(*Mva*I), *Eco*RV, *Hind*III, *Hpa*I, *Pst*I, *Pvu*II(*Cfr*6I), and *Sal*GI. The analysis showed that all of the plasmids isolated from *E. carotovora* strains had unique nucleotide sequences. Figure 3 exemplifies the restriction fragments of two plasmids isolated from strains 48A and 36 (pCA25 and pAT52, respectively), which were digested using the endonucleases *Hpa*I, *Pvu*II, and *Eco*RV. The restriction analysis allowed the molecular masses of some small plasmids to be determined with sufficient accuracy (Fig. 1). However, this method gave inaccurate results for the high-molecular-weight plasmids that were isolated from strains 13A, 33A, C366, and g217 (Figs. 1 and 2) and especially for the plasmids that were isolated from strains with several different plasmids, such as strain 13A (Fig. 1, lane *10*). For this reason, the molecular masses of most plasmids were determined by electrophoresis with the known plasmids, pTi-C58, F, RP4, RSF1010, and pUC18 [12] (Fig. 4).

Table 1 summarizes the results of the determination of the molecular masses of *Erwinia* plasmids expressed in MDa and kbp. It can be seen that about 30% of the *E. carotovora* strains studied have plasmids from 2.5 to 129 kbp in size. The frequency of plasmid occurrence was higher for *E. carotovora* subsp. *atroseptica* (4 of the 6 strains of this subspecies contained plasmids) than for *E. carotovora* subsp. *carotovora* (only 12 of the 46 strains of this subspecies contained plasmids). Of interest is the fact that each of the *E. carotovora* subsp. *atroseptica* strains studied contained only one plasmid, whereas the *E. carotovora* subsp. *carotovora* strain 13A bore 5 different plasmids, strain 35A bore 4 plasmids, and each of the strains 33A, C366, 69A, 66A, and 55A of this subspecies bore 2 different plasmids (Table 1). In this respect, *E. carotovora* subsp. *carotovora* is similar to the species *E. herbicola* [8]. The physiological role of the presence of several plasmids in erwinia cells is as yet poorly understood [2].

Strains C366 and 33A contained both small (4.3 and 5.3 kbp) and large (129 kbp) plasmids. The plasmids of the *E. carotovora* subsp. *carotovora* strains 13A, 33A, and C366 of different origin and of the *E. carotovora* subsp. *atroseptica* strain g217 [9] had the same size, 129 kbp (Fig. 4 and Table 1). Two *E. carotovora* subsp. *atroseptica* strains, 39A and g152, also had plasmids (pAT33 and pAT58) close in size (about 19 kbp). The restriction analysis of these two plasmids showed that they are not identical with respect to the number and position of the restriction sites of the endonucleases *Eco*RV, *Hind*III, and *Hpa*I. At the same time, the three

Table 1. Main characteristics of the cryptic plasmids of *E. carotovora*

Strain	Bacterio-cinotype*	MC group*	Plasmid	Molecular mass**, MDa		Plasmid length, kbp
				I	II	
E. carotovora subsp. *carotovora*:						
48A	1	I	pCA 25	6.4	–	9.8
B566	1	V	pCA 47	6.4	–	9.8
M2-4	2	III	pCA 50	3.5	3.5	5.3
52A	1	VI	pCA 35	–	2.5	3.8
J2	2	I	pCA 44	–	1.6	2.5
33A	2	III	pCA 16-1	–	84.0	129
			pCA 16-2	3.5	3.3	5.3
C366	1	V	pCA 42-1	–	84.0	129
			pCA 42-2	–	2.8	4.3
69A	1	II	pCA 54-1	–	8.7	13.4
			pCA 54-2	–	4.0	6.2
66A	2	V	pCA 18-1	–	7.8	12.0
			pCA 18-2	–	4.5	6.9
55A	1	I	pCA 19-1	6.4	6.6	9.8
			pCA 19-2	3.1	3.2	4.8
35A	1	I	pCA 12-1	–	8.5	13.1
			pCA 12-2	–	6.0	9.2
			pCA 12-3	4.4	4.1	6.8
			pCA 12-4	3.3	3.2	5.0
13A	1	IV	pCA 6-1	–	84.0	129
			pCA 6-2	–	31.0	47.7
			pCA 6-3	–	8.9	13.7
			pCA 6-4	–	3.6	5.5
			pCA 6-5	–	3.2	4.9
E. carotovora subsp. *atroseptica*:						
g217	2	VI	pAT 57	–	84.0	129
39A	1	VI	pAT 33	12.2	–	18.8
g152	1	VI	pAT 58	12.0	–	18.5
36	1	V	pAT 52	3.55	3.55	5.46

*After Thiry [8] and Gorb and Tovkach [9]. ** The molecular mass of plasmid DNA was determined from the data of restriction analysis (column I) and of electrophoresis with the reference plasmids (column II). "–" stands for "not determined."

plasmids (pCA25, pCA19-1, and pCA47) that were isolated from strains 48A, 55A, and B566, close in their origin, not only had similar sizes (9.8 kbp) (Table 1) but were also homologous in the number and position of restriction sites.

Most of the plasmids detected in *E. carotovora* were small (3.7 to 13.7 kbp). The extrachromosomal DNA isolated from the *E. carotovora* subsp. *carotovora* strain J2 was so small (2.5 kbp) that it can hardly be a fully functional plasmid. One of the five plasmids of the *E. carotovora* subsp. *carotovora* strain 13A, plasmid pCA6-2, had the size (48 kbp) that is atypical of the other erwinia strains studied.

Earlier studies showed that *E. carotovora* strains fall into two non-overlapping bacteriocinotypes [11]. The bacteriocinotype 1 strains are characterized by a weak sensitivity to macromolecular carotovoricins (MCs) and some of them are susceptible to the killer activity of the *E. coli* phages T2, T4, and P1. Of interest is the fact that most of the *E. carotovora* strains bearing several plasmids belong to bacteriocinotype 1 (Table 1). It should be noted that the plasmid composition of these

Table 2. Relationship between the presence of plasmids in *E. carotovora* strains and their resistance to antibiotics

	Number of strains tested	Percentage of antibiotic-resistant strains				
		Pnc	Crb	Amp	Ery	Ole
Plasmid-bearing strains	16	56	44	81	25	94
Plasmid-free strains	32	67	66	88	28	100

Note: The concentration of antibiotics was 10 μg/disk.

strains does not correlate with their bacteriolytic [10] or pectinolytic [9] activity.

Investigation of plasmid stability showed that the collection *Erwinia* strains stored for 15 years without subculturing do not contain plasmid-free clones. Treatment with mitomycin C and acridine orange and cultivation at elevated temperatures (38°C for *E. carotovora* subsp. *carotovora* strains and 36°C for *E. carotovora* subsp. *atroseptica* strains) did not lead to plasmid elimination with a noticeable frequency. *Erwinia* strains also retained their plasmids even after multiple subculturing in LB broth or on solid A medium with pectin. The only exception was *E. carotovora* subsp. *carotovora* strain 13A, which lost its atypical plasmid pCA6-2 after three passages on the latter medium. The comparative study of strain 13A and its clones lacking plasmid pCA6-2 with respect to phytopathogenicity, resistance to antibiotics and carotovoricins, ability to grow on polypectate and pectin, and some other properties showed that the only difference between such clones and the parent strain lies in the increased synthesis of MCs in plasmid-lacking clones. For instance, clone 13A-6/15 lacking plasmid pCA6-2 (Fig. 4, lane *7*) produced two to three times more biologically active MCs than the parent strain 13A.

The investigation of the antibiotic resistance of 48 *E. carotovora* strains, either containing plasmids or not (16 and 32 strains, respectively), showed that they fall into nine groups differing in the susceptibility to ampicillin (Amp), carbenicillin (Crb), erythromycin (Ery), penicillin (Pnc), and oleandomycin (Ole). The largest group included 22 strains, most of which were found to be resistant to erythromycin and contain plasmids. The statistical analysis of the results presented in Table 2 showed that the cryptic plasmids of *E. carotovora* are not responsible for the resistance of this species to penicillin (Pnc, Crb, and Amp) and macrolide antibiotics (Ery and Ole) and, therefore, are not R factors.

Thus, about 30% of the *E. carotovora* strains studied have one or several (up to five) cryptic plasmids from 2.5 to 129 kbp in size, which are not responsible for the antibiotic resistance of these strains, i.e., they are not R factors. Three *E. carotovora* subsp. *carotovora* strains and one *E. carotovora* subsp. *atroseptica* strain bear large 129-kbp plasmids, which may play an important part in the ecology of phytopathogenic pectinolytic erwinia.

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