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PHYTOPATHOGENIC BACTERIUM *PECTOBACTERIUM CAROTOVORUM* CRYPTIC PLASMIDS DISTRIBUTION

Information on the extrachromosomal elements occurrence in phytopathogenic bacterium Pectobacterium carotovorum is insufficiently presented in modern scientific literature. Data on the pectobacteria plasmid content are random. ***The aim*** *was to study the Pectobacterium carotovorum plasmids spectra, cryptic plasmids distribution and general characteristics.* ***Materials and methods****. Plasmid spectra of 54 strains of different origins were studied. Standard hot alkaline Kado and Liu method was used to isolate plasmids DNA [Kado C.J., Liu S.-T. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 1981; 145 (3): 1365–1373].* ***Results****. It was found that 16 strains contained plasmids of various sizes. Isolated plasmids belonged to four discrete size classes: 2.5–6.8, 9.8–16.7, 47.7–64.5 and 129 kb. Approximately 50 % of the isolated P. carotovorum plasmids belonged to the second discrete size class with a size of 8.7–10.4 kb. Four large 129 kb P. carotovorum plasmids had unique primary DNA sequence according to results of restriction analysis.* ***Conclusions****. Pectobacteria plasmids isolation results correlate with data obtained earlier that 30 % of strains contained plasmids [Tovkach FI. [Isolation and preliminary characterization of cryptic plasmids from Erwinia carotovora]. Microbiology. 2001; 70 (6): 804–810. Russian]. Strains` plasmid maintenance was associated with environmental and ecological niches where bacteria persisted. These extrachromosomal DNAs may present silent "selfish", and probably prophage, replicons.*

K e y w o r d s: Pectobacterium carotovorum, cryptic plasmids, restriction analysis, ecology, species` plasmids spectra

Extrachromosomal DNAs are widely spread among *Enterobacteriaceae* family members and play an important role in the bacteria ecology and pathology [12]. Phytopathogenic bacterium *Pectobacterium carotovorum* forms a complex taxon which includes strains with different phenotypic, biochemical, genetic and plant-pathogen interactions characteristics, which allows dividing this species to several subspecies [11, 13].

Plasmid spectrum (or profile) is an important species characteristic, based on the specific set of plasmids in the cell. Strains may lose some of the plasmids, but species typical basic set remains unchanged [6].

Pectobacteria strains plasmids spectra are strictly species specific for this bacteria species in nature. The circular DNA plasmids presence was revealed for most bacterial pathogens however, for a significant number of identified plasmids functions remained unclear, which is also true for pectobacteria cryptic plasmids. Usually plasmids make up on between 2 % and 30 % of the total bacterial genome in size [10, 14].

Pectobacterium genus bacteria can inhabit the plants surface, soil, water as symbiotic microorganisms, but mostly they are host plants pathogens. *P. caro-*

tovorum subsp. *carotovorum* (Pcc) can cause soft rot on cabbage, potato and carrot roots and tubers and closely related *P. carotovorum* subsp. *atrosepticum* (Pca) can cause plants "black leg" [8, 10].

Autonomous genetic elements involvement in the formation of new bacterial variants by horizontal pathogenicity gene transfer is the undisputed scientific fact of modern bacteriology. Due to the genes transfer between related bacteria, that inhabit a particular ecological niche, their adaptive capacity is extended, and biodiversity is formed. *P. carotovorum* genetic determinants responsible for adaptational ability and pathogenic potential associated with plasmids or phages pathogenicity islands still have not been identified [11, 13, 14].

The research was carried out to study plasmids spectra, distribution and characteristics of *P. carotovorum* strains cryptic plasmids.

Materials and methods. Plasmid profiles of 54 *P. carotovorum* strains of different origins (Ukraine, Belarus, Russia, Armenia, Romania, Czech Republic, Belgium) were studied: *P. carotovorum* subsp. *carotovorum* – 48A, 75, 144 a, 482 E, 741, 808 a, 7869, NCPPB 550, 718, 566 ВКМ, 246, 915, 184, 2, 48П, 53П, 91П, 921, 2054, ATCC 27388 = NCPPB 1065, G 147, G 117, 258, 133, 5, 180, 495, ATCC 15713^T = NCPPB 312^T (T-type strain), NCPPB 438, SR165, E193, 162, 209, Cc 110, 33A, 13A/15, 4', 9', 10', 11', 16', 18', 3', 15', 23'; *P. carotovorum* subsp. *atrosepticum* – 58A, NCPPB 549^T = ATCC 33260^T, 9Ф, g217, 5A, 37A, 40A, 46A, 194–8; *E. carotovora* subsp. *«toxica»* 47 a, K-47. *Agrobacterium tumefaciens* C58 pTi-C58 plasmid (188 kb) and *Escherichia coli* K12 F plasmid (100 kb) and RP4 plasmid (60 kb), *E. coli* J53 were used as markers in determining the large extra-chromosomal DNAs size.

A standard hot alkaline Kado and Liu method was used to isolate plasmid DNA [9], but with an important modification. Reproducible results were obtained due to the method`s adequacy and universality. However, the *P. carotovorum* plasmid DNA isolation required two- or threefold centrifugation for the aqueous and phenolic phases separation due to the strains mucous material presence after several repeated mixings with phenol. In rare cases, it was not possible to isolate strain`s plasmid due to the cells` high nuclease activity. Also, the pectobacteria plasmids isolation was followed by chromosome less degradation, which affected the track purity during electrophoresis.

P. carotovorum plasmids isolation using Kado and Liu method was adequate, and thus obtained plasmid DNAs were suitable for further molecular genetic studies, including restriction analysis. *Hpa*I, *Bgl*I, *Sal*I, *Eco*RI, *Eco*RV and *Pst*I endonucleases were used for restriction analysis. Plasmid copy number was determined using an analytical transilluminator «BioRad Molecular Imager Doc XR + imaging system» and «Quantity One» program.

Results. Comparison of plasmid DNA samples isolation from bacterial cells grown on solid and liquid nutrient media showed, that cells grown on liquid medium contained more plasmid material. It was found that *P. carotovorum* cells grown on 1 % pectin liquid selective medium contained more plasmid DNA and these samples` bacterial chromosome eliminated better. However, it was true only in case of small plasmids isolation. *P. carotovorum* large plasmids` samples, isolated from cells grown on pectin, contained minimal amounts of the chromosomal DNA impurities compared with samples isolated from cultures grown on plates [2].

It was found that 16 of 54 tested strains contained plasmids of various sizes.

Isolated plasmids belonged to four discrete size classes: 2.5–6.8, 9.8–16.7, 47.7–64.5 and 129 kb. Approximately 50 % of the isolated *P. carotovorum* plasmids belonged to the second class and had a discrete size 9.8 kb. These plasmids copy number was about 8–13 molecules per cell. Next by the occurrence frequency went large 129 kb plasmids. They were found in *P. carotovorum subsp. carotovorum* 194–8, NCPPB 312^T, 33A and *P. carotovorum subsp. atrosepticum* NCPPB 549^T and g217. Plasmids 47.7 and 64.5 kb in size had sporadic occurrence. 47.7 kb plasmid was isolated from NCPPB 549^T = ATCC 33260^T strain, and 64.5 kb plasmid was isolated from NCPPB 312^T = ATCC 15713^T strain (Fig. 1, 2).

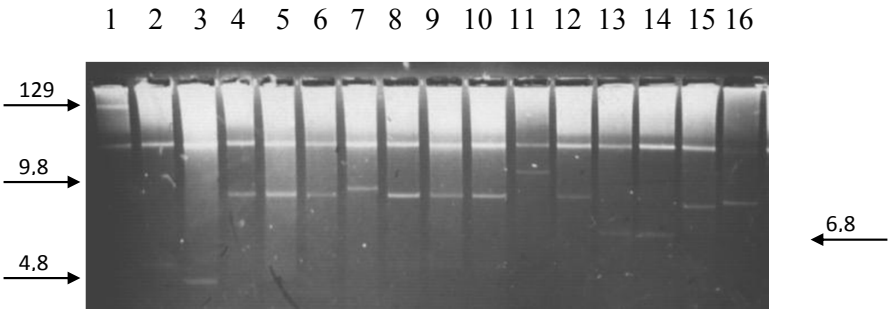


Fig. 1. *P. carotovorum* 3.8 – 16.7 kb plasmids electrophoregram: 33A (1), 495 (2), 3` (3), 2 (4), 75 (5), 48A(pCA25) (6), 921 (7), G147 (8), 718 (9), 566 BKM (10), 48A(pCA25::Tn9) (11), 246 (12), 184 (13), 16` (14), 23` (15) and *E. coli* (pLOF; 9,9 kb) (16)

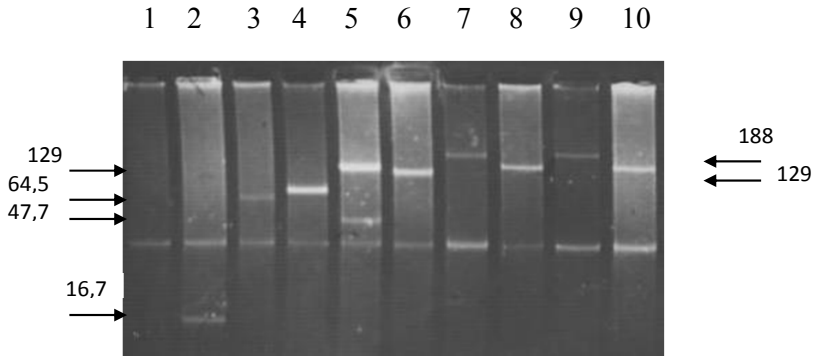
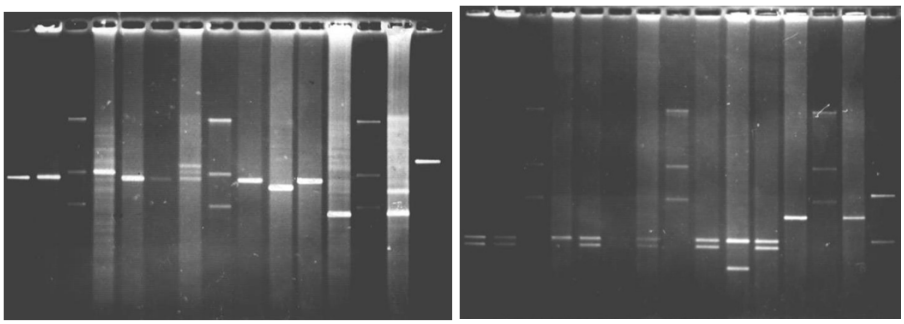


Fig. 2. *P. carotovorum* subsp. *carotovorum* large plasmids electrophoregram: 194–8 (1), NCPPB 312^T (4), 33A (129 kb) (10); *P. carotovorum* subsp. *atrosepticum*: 9Ф (2), NCPPB 549^T (5), g217(8); *E. coli*: J53 (RP4) (3), K12 (F) (6); *A. tumefaciens*: C58 (pTi-C58) (7, 9)

P. carotovorum 48A(pCA25), 2, 75, 921, G 147, 718, 566 BKM, 48A(pCA25::Tn9), 246, 184 strains extrachromosomal DNAs restriction analysis via *Hpa*I and *Eco*RV endonucleases showed that these genetic elements had restriction sites homology. The difference in the restriction fragments sizes resulted only from the original difference in plasmids` sizes due to the deletion-insertion mutations type presence (Fig. 3).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



*Hpa*I *Eco*RV

Fig. 3. *P. carotovorum* plasmids *Hpa*I and *Eco*RV restriction fragments electrophoregram: 2 (1), 75 (2), 921 (4), G147 (5), 718 (6), 566 ВКМ (7), 246 (9), 23` (10), 48A(pCA25) (11), 184 (12), 16` (14), 48A-7/4b (pCA25::Tn*9*) (15); control 3, 8, 13— λ/*Hind*III

Four *P. carotovorum* large plasmids restriction analysis with *Sal*I, *Hpa*I and *Eco*RI endonucleases revealed that each large plasmid DNA had a unique primary sequence (Table 1). Due to the several plasmids presence in the strains only the upper electrophoregram fragments ranging from 25 to 9 kb in size, which presented the large plasmids restriction fragments, were compared. It was established that pCA16-1, pCA42-1 и pCA549-1 plasmids DNAs had restriction sites for each of the three restriction endonucleases used (*Sal*I, *Hpa*I and *Eco*RI). The results indicated that the large plasmids restriction patterns were unique. Thus, unlike *P. carotovorum* plasmids of about 10 kb in size with a similar restriction patterns, all the studied large plasmid were presented by unique sequences.

Table 1

Large plasmids of *P. carotovorum* strains

Strain	Plasmid	Molecule length, kb
33A	pCA16-1	129
	pCA16-2	5.3
NCPPB 549^T	pCA 549-1	129
	pCA 549-2	47.7
NCPPB 312^T	pCA 312-1	64,5
	pCA 312-2	3.8
C366	pCA 42-1	129
	pCA 42-2	4.3

P. carotovorum strains plasmids presence screening revealed that most of the small extrachromosomal DNAs had a size of 9.8 kb or close to it (Fig. 4). One of the most studied *P. carotovorum* genetic elements is pCA25 plasmid [1] also having a size of 9.8 kb. The previous data support the suggestion of its prophage nature [1, 4, 5]. pCA25 plasmid variant – pCA25::Tn*9* – containing the Tn*9* transposon was used for physical mapping. As a result of restriction analysis, it was found that the transposon integrated into a specific locus on a plasmid pCA25 DNA. Similarly, transposons integrate into prophage genomes` non-essential regions. Plasmid pCA25 size is close to the size of the *E. coli*

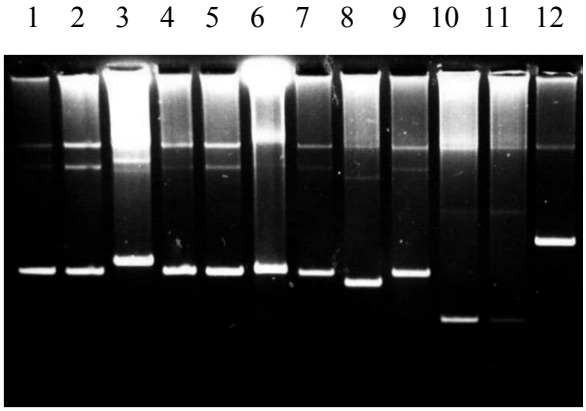


Fig. 4. *P. carotovorum* plasmids DNA electrophoregram: 2 (1), 75 (2), 921 (3), G147 (4), 718 (5), 566 BKM (6), 246 (7), 23` (8), 48A(pCA25) (9), 184 (10), 16` (11), 48A 7/4b (pCA25::Tn*9*) (12)

circular prophage P4 (11.6 kb) [1]. In addition, plasmid pCA25 deletion and insertion variants, isolated from other strains, have genome size changes detected in the same region, where transposon Tn*9* insertion occurs in the strain 48A7/4b and its clones.

All three *Hpa*I sites were located in a short distance from each other, thus resulting in one large and two smaller fragments appearance (Table 2). *Bgl*I site was located in the area between the *Hpa*I sites (Table. 2). These *Bgl*I and *Hpa*I sites were located within *Eco*RV A fragment, in just a short distance from one of three *Eco*RV sites. It was found that the Tn*9* transposon integrated pCA25 DNA in the *Eco*RV B fragment, also in a short distance from the *Eco*RV restriction site. Transposon Tn*9* insertion only into plasmid pCA25 DNA`s certain region was confirmed by the study of a significant plasmid pCA25::Tn*9* clones number, which were independently obtained. *Eco*RI and *Pst*I sites were not found on pCA25 plasmid DNA. There is one *Eco*RI site CAT gene located on the Tn*9* transposon DNA in plasmid pCA25::Tn*9*, and two *Pst*I sites located within the Tn*9* transposon IS1 sequences.

Table 2

Plasmids pCA25 and pCA25:: Tn*9* DNA restriction fragments sizes

Fragment	Hydrolysis option									
	*Bgl*I + *Eco*RI	*Eco*RI + *Hpa*I	*Hpa*I + *Bgl*I	*Bgl*I + *Pst*I	*Eco*RI + *Pst*I	*Hpa*I + *Pst*I	*Bgl*I + *Eco*RI + *Pst*I	*Eco*RV + *Bgl*I	*Eco*RV + *Hpa*I	*Eco*RV + *Eco*RI
pCA25										
A	9.8	9.2	9.2	9.8	–	9.2	9.8	4.55	3.2	4.7
B	–	0.4	0.25	–	–	0.4	–	4.5	4.5	4.5
C	–	0.2	0.2	–	–	0.2	–	0.65	0.65	0.65
D	–	–	0.15	–	–	–	–	0.15	–	–
pCA25:: Tn*9*										
A	8.3	8.9	11.4	8.3	10.5	8.8	7.9	4.5	3.2	4.7
B	4.1	0.4	0.35	2.7	1.9	6.8	2.7	7.1	7.1	5.3
C	–	0.2	0.2	1.7	–	4.3	1.9	0.65	0.65	0.65
D	–	3.0	0.15	–	–	–	–	0.15	0.45	1.85

Note: Numbers indicate the fragments` size in kb .; "–" – the absence of fragment or fragment was not identified.

Discussion. Pectobacteria plasmids isolation results coincide with earlier data showing that 30 % of strains contained plasmids [7].

A comparative study of plasmids intra-species distribution by molecular weight for two representatives of the *Enterobacteriaceae* family showed that *E. coli* and *P. carotovorum* strains plasmids profiles differed greatly (Fig. 5, 6) [12].

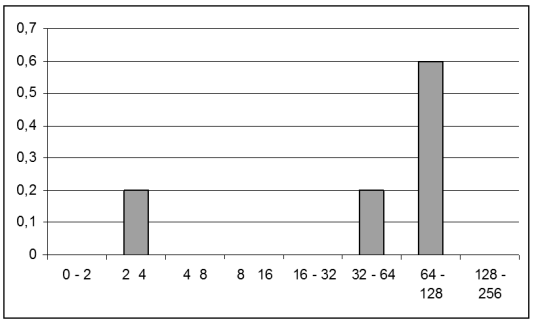


Fig. 5. *E. coli* strains plasmid profiles

The most common *E. coli* strains plasmids were falling in a group with the size range 64–128 kb. This feature differed from the *P. carotovorum* subsp. *carotovorum* (Pss) and *P. carotovorum* subsp. *atrosepticum* (Psa) plasmid profiles, which plasmids, falling into a group of 64–128 kb, were the exception rather than regularity (Fig. 5). The pectobacteria characteristic indicator was the large 129 kb plasmids` presence, that were not found in *E. coli* strains. Furthermore, pectobacteria plasmids also fall within the size ranges from 4 kb up to 32 kb, that was the most typical for Pcc strains and not typical for all *E. coli* strains (Fig. 6).

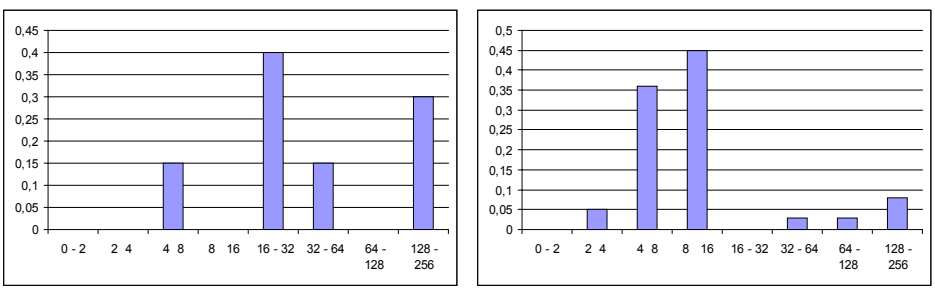


Fig. 6. *P. carotovorum* subsp. *atrosepticum* (Pca) and *P. carotovorum* subsp. *carotovorum* (Pcc) strains plasmid profiles

It was discovered that *P. carotovorum* plasmids of the same size class were found in strains isolated from a particular ecological niche (Table 3). Half of the strains carrying 9.8 kb plasmid or its natural insertion-deletion variants were from Russian collections and obtained on the territory of Russia. The rest of the strains of this group were identified on the territory of Ukraine, Armenia and Romania. Pectobacteria strains from Canada carried no plasmids. With a

single exception, strains, that were isolated on the territory of Belarus, also carried no plasmids. Large plasmids were detected in the strains of the Belgian and Czech collection of microorganisms (Table 3).

Table 3

Plasmid composition of *P. carotovorum* strains

Strain	Plasmid	Size, kb	Geographic area
75	pCA 75	9.8	Russia
718	pCA 718	9.8	Russia
566 ВКМ	pCA 566	9.8	Russia
246	pCA 246	9.8	Russia
2	pCA 2	9.8	Russia
184	pCA 184	6.8	Ukraine
921	pCA 921	10.4	Armenia
G147	pCA 147	9.8	Romania
495	pCA 495	5.8	Russia
NCPPB 312^{T}	pCA 312-1 pCA 312-2	64.5 3.8	Czech Republic
NCPPB 549^{T}	pAT 549-1 pAT 549-2	129 47.7	Belgium
9 Ф	pAT 9	16.7	Russia
3`	pCA 3`	4.8	Ukraine
16`	pCA 16`	6.8	Ukraine
23`	pCA 23`	8.7	Ukraine
89^{++}	pCA 89	15.5	Belarus

The most common group of extrachromosomal elements identified in *P. carotovorum* were 9.8 kb plasmids and their insertion-deletion variants. The second most common group were 129 kb plasmids. This group is of a keen interest, but these elements study has certain difficulties, first of all associated with the isolation of large plasmids DNA avoiding gaps in their molecules. Restriction analysis showed that, despite the same size, 129 kb plasmids differ in restriction patterns [3]. Moreover, strains containing large plasmids were isolated from different ecological niches.

Quite the opposite situation was observed with 9.8 kb plasmids. Half of the strains containing these plasmids belonged to the same ecological niche (Table 3). We assume that these *P. carotovorum* strains` different origins and large plasmids` primary sequences data mismatch may be due to the fact that the large extra-chromosomal DNAs play an important role in the pectobacteria pathogenicity formation towards certain host-plants in their respective ecological niches.

P. carotovorum cryptic plasmids pCA25 and pCA25::Tn*9*, that represented this bacterium most common extra-chromosomal DNA size class, restriction maps were built as a physical mapping result. The transposon marker in the plasmid made possible the restriction sites mutual position specification and Tn*9* transposon location within plasmid DNA [4, 5].

Thus, plasmid maintenance is common among phytopathogenic bacteria *P. carotovorum* strains. Pectobacteria plasmids isolation showed that 30 % of strains contained plasmids of different sizes – from 2.5 to 129 kb. Strains were also characterized by maintenance of two or more plasmids of different sizes

in one cell. Strains plasmid maintenance was associated with environmental niches where bacteria persisted. Pectobacteria extrachromosomal DNAs are the objects of interest as they may present silent prophage replicons. This study creates the role and importance determination prospect of the autonomous genetic elements in the bacteria *P. carotovorum* ecology and physiology.

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ПОШИРЕННЯ КРИПТИЧНИХ ПЛАЗМІД ФІТОПАТОГЕННОЇ БАКТЕРІЇ *PECTOBACTERIUM CAROTOVORUM*

У сучасній науковій літературі представлено недостатньо інформації щодо наявності позахромосомних елементів у важливої фітопатогенної бактерії *Pectobacterium carotovorum*. Дані про плазмідоутримання пектобактерій носять випадковий характер. **Метою** дослідження було вивчення плазмідних спектрів, їхнього поширення та загальна характеристика криптичних плазмід *P. carotovorum*. **Матеріали та методи.** У ході дослідження було проведено скринінг плазмідних спектрів 54 штамів різного походження. Для виділення плазмідних ДНК використовувався стандартний лужний метод Кадо і Ліу [*Kado CJ, Liu S-T.* Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 1981; 145 (3): 1365–1373]. **Результати.** У ході дослідження було встановлено, що в даній індивідуальній виборці 16 досліджених штамів містять плазміди різних розмірів. Виявлені плазміди пектобактерій належать до чотирьох дискретних розмірних класів: 2,5–6,8, 9,8–16,7, 47,7–64,5 і 129 т.п.н. Приблизно 50 % виділених плазмід *P. carotovorum* відносяться до другого дискретного класу і мають розмір 8,7–10,4 т.п.н. У результаті рестрикційного аналізу великих плазмід з однаковим розміром 129 т.п.н. чотирьох різних штамів *P. carotovorum* виявлено, що ДНК кожної плазміди має унікальну первинну послідовність. **Висновки.** Результати з виявлення плазмід пектобактерій збігаються з отриманими раніше даними про те, що 30 % штамів пектобактерій містять плазміди [*Tovkach FI.* [Isolation and preliminary characterization of cryptic plasmids from *Erwinia carotovora*]. Microbiology. 2001; 70 (6): 804–810. Russian]. Плазмідоутримання штамів пов'язане з екологічними нішами, в яких персістують бактерії. Позахромосомні ДНК пектобактерій можуть являти собою мовчазні «егоїстичні», можливо профагові, реплікони.

Ключові слова: Pectobacterium carotovorum, криптичні плазміди, рестрикційний аналіз, екологія, плазмідний спектр виду.

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РАСПРОСТРАНЕНИЕ КРИПТИЧЕСКИХ ПЛАЗМИД ФИТОПАТОГЕННОЙ БАКТЕРИИ *PECTOBACTERIUM CAROTOVORUM*

В современной научной литературе представлено недостаточно информации относительно встречаемости внехромосомных элементов у важной фитопатогенной бактерии *Pectobacterium carotovorum***.** Данные о плазмидном содержании пектобактерий носят случайный характер. **Целью** исследования было изучение плазмидных спектров, их распространения и общая характеристика криптических плазмид *P. carotovorum*. **Материалы и методы.** В ходе исследования был проведён скрининг плазмидных спектров 54 штаммов различного происхождения. Для выделения плазмидных ДНК использовался стандартный щелочной метод Кадо и Лиу [*Kado CJ, Liu S-T.* Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 1981;145(3):1365–1373]. **Результаты.** В ходе исследования было установлено, что в данной индивидуальной выборке 16 исследованных штаммов содержат плазмиды различных размеров. Обнаруженные плазмиды пектобактерий принадлежат к четырём дискретным размерным классам: 2,5–6,8, 9,8–16,7, 47,7–64,5 и 129 т.п.н. Приблизительно 50 % выделенных плазмид *P. carotovorum* относятся ко второму дискретному классу и имеют размер 8,7–10,4 т.п.н. В результате рестрикционного анализа больших плазмид с одинаковым размером 129 т.п.н. четырёх различных штаммов *P. carotovorum* выявлено, что ДНК каждой плазмиды имеет уникальную первичную последовательность. **Выводы.** Результаты по выявлению плазмид пектобактерий совпадают с полученными ранее данными о том, что 30 % штаммов пектобактерий содержат плазмиды [*Tovkach FI.* [Isolation and preliminary characterization of cryptic plasmids from *Erwinia carotovora*]. Microbiology. 2001; 70 (6): 804–810. Russian]. Плазмидосодержание штаммов связано с экологическими нишами, в которых персистируют бактерии. Внехромосомные ДНК пектобактерий могут представлять собой молчащие «эгоистичные», возможно профаговые, репликоны.

Ключевые слова: Pectobacterium carotovorum, криптические плазмиды, рестрикционный анализ, экология, плазмидный спектр вида.

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