UDK 579.26

N. V. Boiko [1], I. I. Chonka [1], V. P. Stefurak [2]

DESTRUCTION AND PHENOL-TOLERANCE MECHANISMS OF *AEROMONAS HYDROFILA* STRAIN, ISOLATED FROM INDUSTRIAL WASTE WATERS

[1] Uzhhorod National University
[2] Ivano-Frankivsk State Medical Academy

The given thesis is devoted to the analysis of bio-destructive features of the *Aeromonas hydrofila* strain, isolated from the industrial discharge of a wood-chemical factory in conditions of model phenol pollution in experiments *in vitro*. It has been shown that *Ae. hydrofila* strain isolated from the polluted discharged water is characterized by its high ability to phenol bio-destruction in experiments *in vitro*. Plasmid conditionality of the selected strain's resistance to antibiotics and phenol was studied, and the structure of its plasmid DNA was explored. The results of multi-enzyme restriction of *Ae. hydrofila*'s plasmid DNA enabled to construct its physical map and grounded the suggestion that obtained strain's resistance to phenol pollution of the environment could be hypothetically encode by three t. n. p. of pAR.

Key words:Aeromonas hydrofila strain, phenol bio-destruction.

Н. В. Бойко[1], І. І. Чонка[1], В. П. Стефурак[2]
[1] Ужгородський національний університет
[2] Івано-Франківська державна медична академія

ДЕСТРУКЦІЯ ТА МЕХАНІЗМИ СТІЙКОСТІ ДО ФЕНОЛУ ШТАМУ *AEROMONAS HYDROFILA,* ВИДІЛЕНОГО ІЗ ПРОМИСЛОВИХ СТІЧНИХ ВОД

Проаналізовано біодеструктивні властивості штаму *Aeromonas hydrofila* в умовах модельного фенольного забруднення. Показано, що *Ae. hydrofila* характеризується високою здатністю до біодеструкції фенолу в експериментах *in vitro*. Доведено плазмідну обумовленість стійкості відібраного штаму до антибіотиків пеніцилінового ряду і фенолу. Вивчено структуру плазмідної ДНК дослідного штаму. За результатами мультиферментної рестрикції досліджуваної плазміди pAR *Ae. hydrofila* побудовано її фізичну карту. Обгрунтовано припущення, що виявлена резистентність штаму *Ae. hydrofila* до фенольного забруднення середовища кодується трьома нуклеотідними послідовностями pAR.

Ключові слова: штам Aeromonas hydrofila, біодеструкція фенолу.

The idea of treatment of high-concentration industrial wastewater against toxicants of both organic and non-organic nature with the help of enzyme-active bacterial cultures has of late become fully realized in practice: that is the way how the polluted discharge is purified from residues of phenol, thiocyanates, cyanides, polycyclic, aromatic hydrocarbons, ammonia, etc. (Members of the Family .., 2002; Phylogenetic relationships .., 2002). The fact of complete recycling of industrial wastewaters that may be used following their deep treatment as irrigation waters in agriculture and even directly discharged into water basins is proved (Yurovska, 1984).

Pseudomonas spp., Thiobacillus thyocyanoxidans, *Th. denitrificans, Micrococcus spp.*, *Bacillus spp.*, *Chromobacterium spp.*, *Sarcina spp.*, *Vibrio spp.* species that are most frequently used as traditional bacterial bio-destructors (Microbial Destruction of Cyanide .., 2000; Microbiological Destruction of Organic .., 1987; Yurovska, 1984) owing to preliminary scrupulous screening of the cultures *in vitro* and/or as a result of natural selection *in situ* of the strains that are most resistant against different pollutants. Water basins and soils contaminated with various toxic substances, are the source of their isolation. The main drawback of industrial use of the latter is their substantial strictness to the conditions of existence, viz.: necessity to maintain the optimum quantity of nutrients, good aeration supply, corresponding temperature scale, etc., thereby the technological process of treatment of polluted water becoming physically and economically more complicated.

Mechanisms of the microorganisms' resistance to toxic substances and abilities of their utilization abilities have not been clarified by now. Profound study of the given phenomenon is of significant theoretical interest. From the other hand, such information is also necessary to construct new highly pollutant-resistant bacterial bio-protectors that, apart from benefiting

from their high destructive potential against toxic substances, would be relatively unpretentious to the conditions of existence.

Search for and selection of activity-direction-specific bacterial bio-destroyers, as well as study of their resistance to pollutants stipulated genetically, must undoubtedly be treated topical for improvement of functioning of the existing water-treatment systems, and for the development of new ones.

The given thesis is devoted to the analysis of bio-destructive features of the *Aeromonas hydrofila* strain, isolated from the industrial discharge of a wood-chemical factory (the settlement of Perechyn, the *Domaradzh* flood, Zakarpatska Oblast, Ukraine) in conditions of model phenol pollution in experiments *in vitro*. Plasmid conditionality of the selected strain's resistance to antibiotics and phenol was studied, and the structure of its plasmid DNA was explored.

METHODS

Aeromonas hydrofila in the titre of $7{,}5 \times 10^9$ CFU/ml were introduced into liquid minimal nutritional medium (MNM) chemically most approximated to the natural conditions of existence of the given bacterial isolate (g/l): NaCl – 5, K_2HPO_4 – 2, Na_2HPO_4 – 2, tap water – 1000 ml. Phenol destruction activity with the given strain was determined by using it in the concentrations of 400 and 1000 mg/l, while the latter had preliminarily been defined as sub-lethal (DL_{80}). Duration of the model experiment was 6 days at 16° C *in vitro*. *Aeromonas hydrofila's* biodestructive abilities against phenol were studied in dynamics every 24 hours by quantitative analysis of the latter's residue in the medium with the use of the method of photometric determination of the mixture of volatile phenols with 4-aminoantipyrine (CND .., 2001).

To investigate the mechanisms of the given strain's resistance, the bacteria had preliminarily been separated from the MNM with phenol by nylon bacterial filters having the diameter of about 1 nm.

Following their re-cultivation, presence of plasmid DNA was checked by alkaline lyses method (Genetic manipulation .., 1985).

A plasmid isolated from the strain under study, was named pAR. It underwent restrictive analysis with the help of the following restrictive-enzymes: BamHI, PstI, XhoI, HindIII, PvuI, EcoRV, EcoRI, ScaI (Maniatis, Frich , Sambrook, 1984). To study the functional abilities of the plasmid isolated, it was transformed (Maniatis, Frich, Sambrook, 1984) into DH5α, an ampicillin-sensitive strain of *E. coli*. The transferred pAR was subject to a repeated restriction with double restrictases to determine its size and construct its physical map.

RESULTS

Growth diagnostics of the *Aeromonas hydrofila* author's strain within the initial hours of the experiment showed that in the liquid MNM, provided adding of different phenol concentrations, the level of CFU/ml of bacteria was reducing significantly, in comparison with the quantity of inoculum introduced primarily (Fig. 1). Abrupt drop of the content of viable cells of the given strain in the MNM was registered during the first 72 hours at phenol concentrations of 400 mg/l. When the toxic agent had a higher load per 1l of the medium (1000 mg/l), similar significant drop of the titres was observed only within the first day. Further daily measurements of the quantity of CFU/ml of the given bacterial strain verified gradual growth of their titres up by 10,1 and 22,3 %, correspondingly, from the concentration initially introduced (Fig. 1).

As seen from Fig. 2, when affected by *A. hydrofila* in the MNM with the phenol content of 400 mg/l, the latter's concentration was insignificantly changing within the initial 24–48 hours. That was caused by the bacteria's pre-adaptation to the given nutrient medium. However, starting from the 3rd day of the experiment, the phenol concentration was steeply reducing down almost to its complete utilizing (84,8 %). Then, the bacterial cells' titre achieved its maximum at $3{,}5 \times 10^8$ CFU/ml.

Genetic analysis of availability of plasmid DNA in the *A. hydrofila* strain under study, made it possible to reveal a small-sized plasmid (≈ 4,4 thousand nucleotide pair, t. n. p.), named pAR, in their cells' cytoplasm. Restrictive analysis of pAR showed (Fig. 3) that no recognition

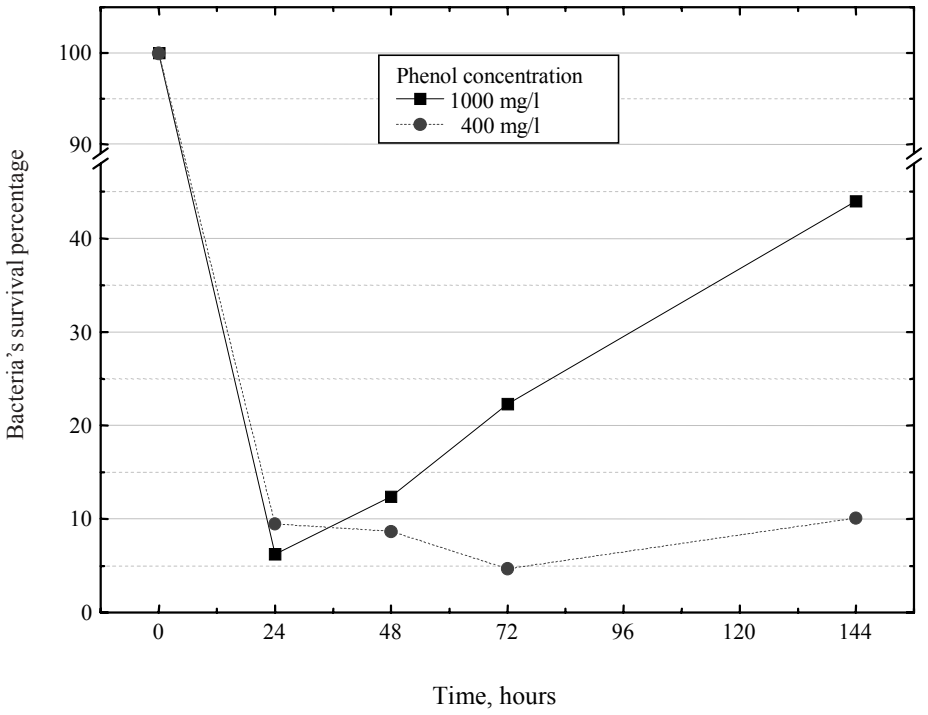

Fig. 1. **Dynamics of the number of *Aeromonas hydrofila* bacterial cells depending upon the quantity of phenol in the MNM**

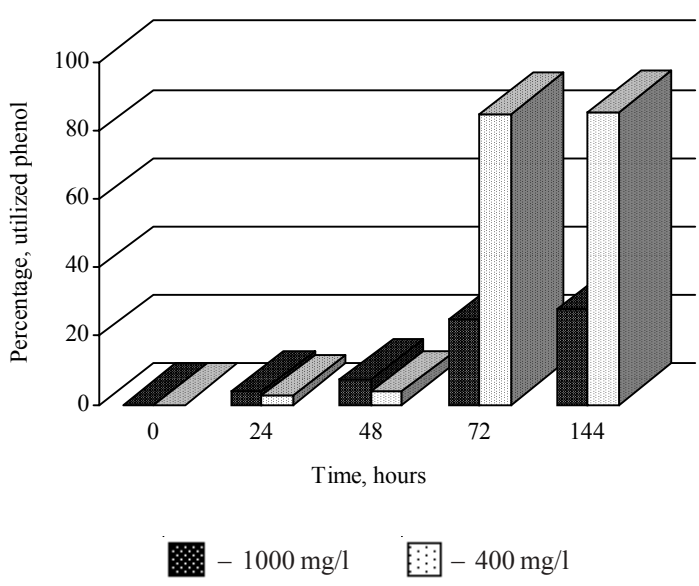

Fig. 2. **Intension of phenol bio-destruction by *Aeromonas hydrofila* culture depending of the formers concentrations in MNM**

sites for EcoRV restrictase were found there, while we found one restriction site for PstI and ScaI, and two sites for each of the following: BamHI, HindIII, and EcoRI. The latter three endonucleases divide pAR to two approximately equal fragments, viz.: 2,1 and 2,3 t. n. p.

Determination of the investigated *A. hydrofila* culture's sensitivity to antibiotics showed its high resistance to carbenicillin and ampicillin**,** not observed in case of the *E. coli* DH5α strain. As a result of the pAR's transformation into the given strain of *E. coli*, the latter's ampicillin- and carbenicillin-resistant clones were obtained. That fact gives us grounds to suggest that the investigated pAR plasmid is able to codify the sign of bacteria's resistance to penicillin-type antibiotics.

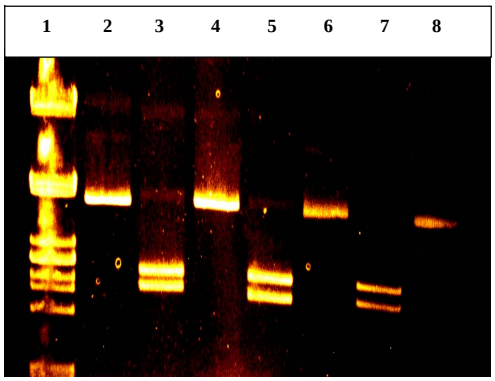

Fig. 3. **Electrophoregram of restrictive fragments of *Aeromonas hydrofila*'s pAR plasmid**: 1 – marker (λ/PstI); 2 – pAR/ScaI; 3 – pAR/EcoRI; 4 – pAR/PstI; 5 – pAR/HindIII; 6 – pAR/EcoRV; 7 – pAR/BamHI; 8 – native pAR

The results of the pAR's treatment with one and two restrictases showed that, with the help of PstI and ScaI enzymes, one ≈ 4,3–4,4 t. n. p.-sized fragment of DNA was forming (Fig. 3, 4). That means that within a pAR molecule, two unique recognition sites for the said restrictases existed. According to pBR322 plasmid map, or other similar plasmids from which pUC18/19,

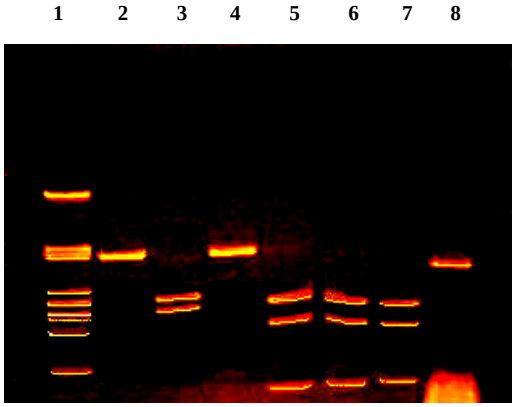

Fig. 4. **Electrophoregram as a result of pAR plasmid restriction: by using of PstI; PstI and ScaI**: 1 – λ/PstI; 2 – pAR/PstI; 3 – pAR/BamHI; 4 – pAR/ScaI-PstI; 5 – pAR/EcoRI-PstI; 6 – pAR/BamHI-PstI; 7 – pAR/HindIII-PstI; 8 – native pAR

pBluescriptIIKS, etc. vectors were constructed, one may guess that in the pAR plasmid, the given sites were located in the *β*-lactamase gene bla responsible for the bacteria's resistance to ampicillin. Approximately 3 t. n. p. in pAR may cause the given strain's resistance to the environmental pollution with phenols. Double restrictase HindIII-PstI subdivided pAR into

3 fragments of approximately 0,6; 1,7; 2,1 t. n. p. each, i. e. the recognition site for HindIII was located 600 n. p. to the left or right from the recognition site for PstI restrictase. Similarly, it was stated that by using of BamHI-PstI and EcoRI-PstI, also three fragments were created (≈ 0,5; 1,8 and 2,1 t. n. p.). Thus, the recognition site for BamHI was located 500 n. p. to the left or right from the recognition site for PstI restrictase. On the basis of the above data, pAR plasmid's physical map has been compiled (Fig. 5).

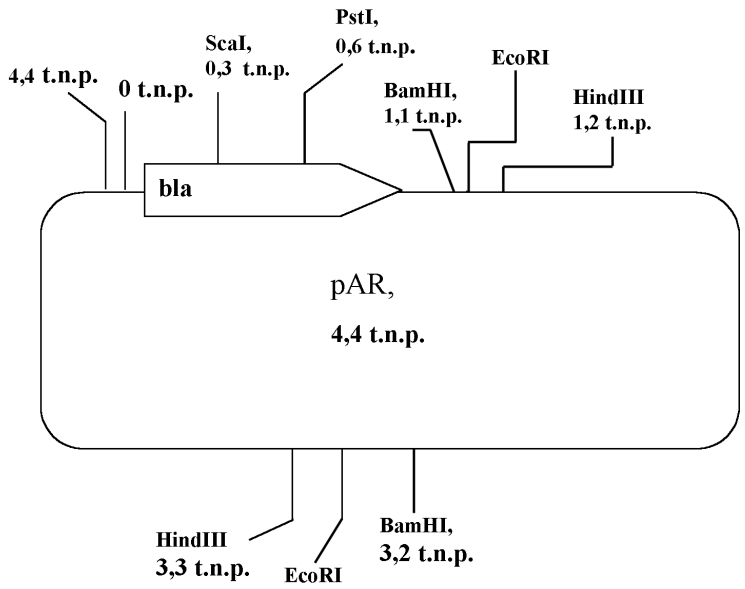

Fig. 5. **pAR plasmid's physical map. Plasmid was isolated from *Aeromonas hydrofila* strain relatively resistant to phenol-type pollution**

DISCUSSION

Aeromonas hydrofila strain relatively resistant to phenol-type pollution from industrial wastewater has been isolated, and its bio-destructive potential and genetic mechanisms of such resistance have been studied. Growth dynamics of the given author's strain, and its phenol-utilizing activity determined *in vitro*, are dose-dependent. In the both cases (exit phenol concentrations in the MNM being 400 and 1000 mg/l), an appropriate drop of the inoculated bacteria's titre was observed, while adaptation to the pollutant's high concentrations (1000 mg/l) was taking place much quicker – within the first day (vs. 3 days at the phenol concentration of 400 mg/l). Further, gradual rise in number of CFU/ml of the given bacterial strain was observed accordingly to $7,6\times10^8$ (400 mg/l) and $1,7\times10^9$ CFU/ml (1000 mg/l). Apparently, that was caused by acceleration of the process of bacterial pre-adaptation and selection of resistant clones affected by more intensive (extreme) environmental pollution.

The question is of the highest interest, viz.: is there any direct relation between fast adaptation and its phenol-recycling ability? In other words, will or not such fast selection ensures increase of the phenol destruction activity in extreme conditions?

It was revealed that *A. hydrofila*'s phenol destruction activity had been insignificant during the initial 24–48 hours (Fig. 2). However, beginning from the 3rd day of the experiment, phenol concentration would be steeply falling almost to its complete utilizing (84,8 %). In addition, the bacterial cells' titre would reach its minimum – $3,5\times10^8$ CFU/ml. Indeed, in the variant of the experiment with critical phenol concentration in the medium (1000 mg/l), growth of the number of *A. hydrofila*'s tolerant forms was already observed on the 2nd day of cultivation, but the utilized phenol then would reach only 7,2 %. The latter index would further be somewhat

growing according to the rise in number of the bacteria's viable forms (Fig. 2), but it became apparent and/or proved that at those pollutant concentrations in the MNM, destruction would be less efficient.

Therefore, growth of *A. hydrofila* titre following a short-time oppression of the bacterial growth may be explained in the both cases by the survival of the resistant clones able to use phenol compounds and dead bacterial biomass as a nutrition source (including carbohydrates). In the variant of the experiment with phenol content of 400 mg/ml, most introduced bacterial cells would perish at the beginning of the experiment (95,3 %), but it was there that the highest percentage of recycled phenol (84,8) was observed. These novel observations demonstrate possibilities to apply *A. hydrofila* for treatment of phenol-polluted wastewater as effective bio-destructive component of complex filter system on zeolites matrix. As we documented previously (Bacteriological assessment .., 2001; Boiko, Dziamko, Chonka, 2001) the using of such environmental materials for purification of industrial polluted water had bright prospect.

We revealed plasmid conditionality of *A. hydrofila*'s resistance that may explain rather fast adaptation of the bacteria to sub-lethal phenol concentrations (1000 mg/l), yet its bio-destruction was in that case insignificant – maximum 24,8 %.

Our data suggest that pAR plasmid under review was shown to be able to codify the sign of the given bacteria's resistance to penicillin-type antibiotics. The results of multi-enzyme restriction of *A. hydrofila*'s plasmid DNA enabled to construct its physical map, according to whose functional analysis approximately 3 t. n. p. of pAR may explain that strain's resistance to phenol pollution of the environment.

CONCLUSIONS

1. The *Aeromonas hydrofila* strain isolated from the polluted discharged water is characterized by its high ability to phenol bio-destruction in experiments *in vitro*.

2. On the 3rd day of cultivation of the investigated culture, the part of the utilized phenol (the introduced amount – 400 mg/l) equaled to 84,8 % under conditions to the maximum approximated to natural.

3. Both growth dynamics of the given author's strain and its phenol-destructive activity are dose-dependent.

4. The given bacterial strain was noted to have plasmid DNA 4,4 t. n. p. approximately.

5. Its plasmid's site of ≈ 1,1–1,2 t. n. p. may be located within ampicillin-resistance gene bla, and it may contain information able to codify the sign of the culture's resistance to penicillin-type antibiotics, and also to be responsible for initiation of replication.

6. 3 t. n. p. in pAR may cause *Aeromonas hydrofila*'s resistance to phenol pollution of the environment.

Acknowledgments

We are very grateful to Docent Victor O. Fedorenko and C.Sc. Bohdan O. Ostash (Department of Genetic, Ivan Franko Lviv National University) for their expert technical assistance in all genetic assays. The authors acknowledge the advice and helpful discussion of Academic Bohdan Matselyukh, the Head of the Department of Division of Microbial Genetic (Institute of Microbiology and Virology, National Academy of Science, Ukraine, Kyiv).

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Надійшла до редколегії 17.11.03