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N. O. Kozyrovska, G. L. Kovtunovych, O. V. Lar, M. V. Kovalchuk, V. V. Negrutska, O. S. Korniichuk, I. S. Rogutskyi, A. P. Alpatov, V. A. Kordyum

Institute of Molecular Biology & Genetics of the National Academy of Sciences

Modeling molecular plant-bacteria interactions for flight experiment

The ability to grow plants in space self-perpetuating gardens is an actual for providing an advanced life support system for humans during extended missions. However, space factors affect expression of the genes regulated by the systems, sensing environmental signals. In space a risk of genetic rearrangements is increased, and some changes in bacterial DNA expected. As a consequence, bacteria may exhibit novel characters, e.g., pathogenicity. During the previous our experience we have determined an increase of internal colonization of the rice roots with bacteria in space flight. To understand the data and to predict acquisition of increased aggressiveness towards the plant-host by bacteria, molecular-genetic plant-bacteria interactions affected with physical factors will be simulated. Genes coding for bacterial pectinases provide a suitable model for studies of well-integrated objectives, concerning plant-bacteria interactions.

INTRODUCTION

Data from flight experiments show that future astronauts can grow multiple generations of plants in space [24]. Such self-perpetuating gardens will be a practical necessity for humans as they explore and colonize the solar system. Hardy space plants could provide fresh food, oxygen, and even clean water for explorers living for long stretches aboard orbiting outposts. Nevertheless, many questions remain how cosmic factors may influence plant growth and development, on the one hand, and microorganisms that are always present both on the surface and in plant interior, on the other hand. Peculiarity of growth and development of bacteria in microgravity being investigated within many years by Ukrainian researchers at the USSR spaceships and stations [11, 12, 18, 25]. Our data exhibited the unusual interrelationships between eu- and cyanobacteria and a plant host in space flight [12, 25]. The increased accessibility of plants to microorganisms may mean that the plants are not only more susceptible to recognized pathogens, but they may also be susceptible to pathogenic colonization by opportunistic pathogens, i.e., organisms that are not normally pathogens to the plant [17]. However, previous experiments concerned to physiology of microorganisms, and molecular mechanisms of their nonstandard functioning under cosmic factors have not been investigated. In the space environment living organisms are under the stress conditions, and there is a risk of genomic alterations [2] and changes in gene expression [1, 31]. Cosmic conditions affect cell microenvironment and signal transduction through membranes [26], and it may result in induction of some genes regulated by the systems, sensing environmental signals, and, as a consequence, bacteria may exhibit novel characters, e.g. phytopathogenicity. It seems to be important to study molecular plant-bacteria interactions in a context of growing plants in space greenhouses for providing an advanced life support system for humans during extended missions away from earth.

The purpose of this research is a theoretical substantiation and experimental examination of a model that can be used for studying some bacterial molecular genetic processes in a confined system (microcosm) in following integrated tasks: (1) a mode of expression of genes encoding pectinolysis in bacteria;

(2) a putative variation of pectinase genes in a response to cosmic factors; (3) a transfer of plasmid/transposon in bacterial populations colonizing a plant. Additional objectives might be incorporated in the same model system.

MODELING MOLECULAR PLANT-BACTERIA INTERACTIONS IN GROUND EXPERIMENTS

Examining a mode of expression of pectinase genes of a plant-associated bacterium in microcosm experiments. Our previous data exhibited the unusual interrelationships between endophytic enterobacteria and a plant host (rice) in a short-term space flight on the Mir Orbital Station [25]. The enlarged intercellular spaces in the plant roots packed by bacteria have been revealed, and it could be a result of the induction of genes, coding for plant cell wall degrading enzymes. It was supposed that plant-cell-wall-degrading enzymes of experimental bacteria were developing activity during space flight. Pectinolytic activity of the bacterium used in the experiment, *Klebsiella oxytoca* [15, 19], was being screened. *K. oxytoca* was able to depolymerize a polymer of polygalacturonic acid pectin, however, the level of activity was low [13]. The mode of expression of the pectinase encoding genes may be different in earth and in flight experiments.

The *pel*X and *peh*X genes from bacterium *K. oxytoca* VN13, encoding a pectate lyase and polygalacturonase, respectively, were isolated by expression in *E. coli* [14, 16]. Nucleotide sequence analysis of the regions containing *pel*X and *peh*X indicated no homology with the other bacterial sequences deposited in public gene banks. Analysis of the deduced PelX polypeptide revealed 77 % of identity to exopolygalacturonate lyase of *Erwinia chrysanthemi* and *Pectobacterium chrysanthemi*, 46 % of *Bacillus halodurans* and 49 % of identity to putative polysaccharide lyase of *Streptomyces coelicolor* A3. PehX disclosed homology to exopolygalacturonases of *Yersinia enterocolitica, Erwinia chrysanthemi* and *Pectobacterium chrysanthemi* with a 52, 50 and 48 % of identity, respectively.

A reporter gene fusion technology will be used to monitor pectate lyase or polygalacturonase gene expression by the quantitative image analysis [3]. We have constructed the translation fusion of the *pel*X promoter with the coding part of the *lac* reporter gene derived from pDK5 [10] (Fig. 1). Comparative studies of expression of the *pel*X-*lac*Z fusion in *E. coli* and *K. oxytoca* VN13, using such inductors as a plant extract or polygalacturonate, showed a low constitutive level of the *pel*X expression in *K. oxytoca* VN13 as well incomplete derepression effected by inductors. It may be feasible to monitor changes in gene expression after low-dose radiation exposure and other physiological stresses in ground experiments. For flight experiments the fluorescent proteins (encoding green or red fluorescent proteins, *gfp* and *rfp*, correspondingly) are the advantageous reporters because leave information about the tagged gene (and organism, accordingly) even the organism is dead, so this reporter system is a suitable for a study of impact of cosmic factors on gene expression after returning experimental material back on earth.

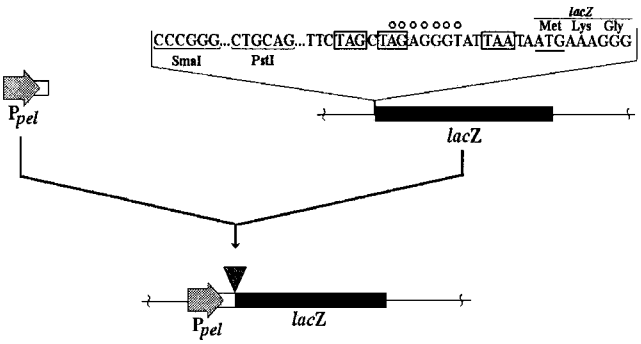

Fig. 1. Scheme of the pelX gene promoter with the lacZ gene coding part fusion. Stop codons that terminate translation marked with frames, the ribosome binding site — with circles. The beginning of the lacZ gene and the translated amino acid sequence is displayed.

Detection of putative variation in the model pelX and pehX genes. The numbers of space flight experiments exhibit the impact of space radiation on living organisms. The experiments performed on board of satellites indicate that the space conditions may enhance the mutation frequency of certain genes in bacteria [7, 8, 30]. The experience of Space Station Mir within 1992—1999 exhibited increase in an overpatching of physiological characters of bacteria and micromycetes that resulted in destroying materials and constructions [27]. Among factors influenced this was permanent low dose cosmic ionizing irradiation. It is difficult to compare ground studies with space experiments because of complexity of the space radiation environment, however, it was concluded that neutrons coursed stronger effect on microorganisms than gamma-rays. The short-term Space Shuttle experiments did not reveal a difference between the space and control bacterial samples [6]. The objective of this study is to establish effect of low dose radiation on genetic diversity of endophytic bacterium *K. oxytoca* in a context of its behavior on/in the plant roots. The idea is to assess genetic

heterogeneity of the *pehX* and *pelX* genes, as well repetitive sequences. For molecular analyses of the assumed changes in the mentioned genes of *K. oxytoca* total DNA will be isolated from flight and ground (control) microcosm. A thermal gradient gel electrophoresis (TGGE) analysis of the *pehX* and *pelX* genes, as well ERIC (enterobacterial repetitive intergenic consensus) elements directly amplified from total DNA, to determine changes in composition of known gene sequences will be used. TGGE is based on the separation of gene sequences directly amplified from DNA by using conserved primers in a denaturing gel according to their melting point [21]. The amplified DNA will be also subjected to PCR-RFLP (restriction fragment-length polymorphism) analysis with primers designed for the pectinase genes.

Simulating the genetic exchange between bacteria in the wheat rhizosphere. Conjugation is an important gene transfer mechanism for bacteria in the rhizosphere [29], and the genes responsible for mating-aggregate formation and DNA transfer are often carried by self-transmissible plasmids. Such conjugative plasmids are known to be capable of recruiting chromosomal genes as well as mobilizing non-self-transmissible plasmids and hence can provide genetic plasticity to bacterial populations. Plasmid transfer between introduced bacteria via conjugation in soil has been unequivocally shown in numerous microcosm ground experiments with different mating combinations, including taxonomically diverse ones. In particular, rhizospheres of crop plants, such as wheat, provide conditions conducive to conjugal plasmid transfer between bacterial inhabitants [28]. However, there is a paucity of knowledge concerning an impact of cosmic factors on conjugation. Exchange of genetic elements and gene transfer between bacteria in confined systems has to be taken into account, and risks for genetically modified organisms (GMO) evaluated. In such systems there is no normal competition for survival, and risk of GMO to populate appropriate niche without selective pressure is a high. Transposon migration between bacteria in the plant rhizosphere of orbital greenhouses may be higher than in the natural environment. Experiments on simulating of plasmid/transposon transfer between bacteria in microcosm and evaluation its migration will be performed, using the model system.

The natural conjugal gene flow in gram-negative bacterial pairs in the wheat rhizosphere in different substrates will be studied in ground experiments. The plasmids were constructed in this study for long-term microcosm experiments. Scheme of the plasmid construction is represented on Fig. 2. Transfer of plasmids pSUP2021 [23] and pSUPARS from *E. coli* to

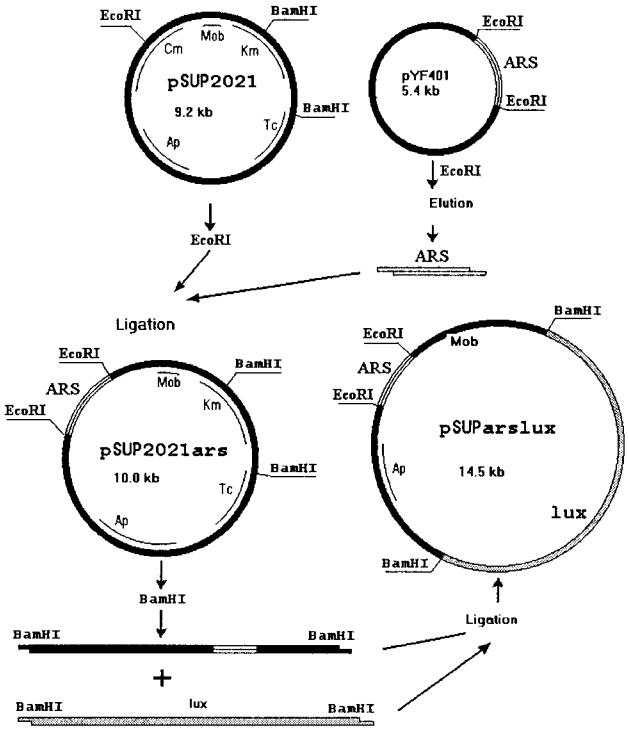

Fig. 2. Scheme of the plasmids pSUPARS and pSUPLux construction. A 1.2 kb fragment of autonomously replicative sequences (ARS) of maize (pYF401, [22]) was inserted into pSUP2021.The resulted plasmid pSUPARS ligated with the *lux* operon of *Photobacterium leiognathi.*

K. oxytoca, Agrobacterium radiobacter, Pseudomonas fluorescens bacteria varied at frequencies of 2×10^{-3}—2.0×10^{-7} transconjugant per donor. A stability of the plasmids after 100 generations in*A. radiobacter* was for pSUPARS — 99 %, pARSLux — 67 %. pSUP2021 segregated from *A. radiobacter* after 20 generations. This data exhibited a high stability of the recombinant plasmids conferring the ARS fragment. In microcosm experiments the plasmids were transferred within a day with frequency $(1—3)\times10^{-5}$ CFU g^{-}. Transposon migration will be simulated among bacteria of the rhizosphere population, using a suicide vectors pCAM120 [32] and pUT/Km [4]. Two-parental matings between *E. coli,* conferring mTn*5*Ss*gus*A20 or Tn*5*/Km, and two strains of bacteria — *K. oxytoca* and *P. fluorescens* have been performed on plates. Frequency of transposition of mTn*5*Ss*gus*A20 and Tn*5*/Km was evaluated in recipient strains as 10^{-6} CFU/ml.

Analysis of endophytic bacterial communities of the plant. Analysis of literature cited revealed data concerning a location of bacteria inside of potato tissue [5, 20] and agronomic crops [33]. Endophytic

bacteria populated interior of roots and stems belong to known already bacterial species as well to nonidentified on a reason of unculturability. Some of isolates have high homology with human pathogens *Staphylococcus* and *Mycobacterium* [33]. Such an unexpected finding has to be taken into account in monitoring bacterial populations in space gardens. On the other hand, endophytes as competitive bacteria can be used for growing plants as probiotics and biofertilizer. For example, the *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state [9]. The use of such a bacterium for plant fertilizing is a profitable in a contrast to mineral fertilizers that weigh and occupy space for storage.

Our data show that composition of the endophytic isolates derived from axenically grown plantlets from tissue culture is likely influenced by the plant inoculation with bacterial component. Since the incidence of endophytic bacteria composition is affected by ecological factors, the impact of microgravity and other cosmic factors on the endophytic bacterial community composition must be studied. The diversity of endophytic bacterial populations of potato is planned to be assessed using a combination of dilution plating of plant macerates and direct PCR-TGGE on the basis of DNA extracted from plants.

Detection of bacteria on/into the plant. Detection of bacteria on/into the plant and estimation of survival of experimental bacteria in the rhizosphere can be performed with PCR DNA fingerprinting, histochemical GUS assay or fluorescent microscopy of infected roots. For ground experiments we used bioluminescence method for estimation of colonization pattern and survival of bacteria in the rhizosphere when colonization pattern of the plant in microcosms can be visualized by naked eyes. Our data exhibited that *K.oxytoca* (pSUPLux) colonized roots of a non-inoculated wheat within 10—12 days (mineral substrate) or 18 days (the agar medium), being introduced into the microcosm with inoculated seeds of a wheat mixed in 1 : 1 with noninoculated ones. The *gus* reporter gene gives information about location of bacteria on/in the plant-host. Transposon mTn5Ss*gus*A20 was used to tag the model bacterial strains. The transposon-conferring *K. oxytoca* and *P. fluorescens* have been selected as indicator bacteria to monitor its behavior under cosmic conditions. Transposon-induced derivatives have been estimated in relations with the plant as described in [32]. On the base of data obtained conclusion has been made that in microcosm in aseptic conditions transposants of *K. oxytoca* and *P. fluorescens* were able to enter the plant roots. Transposants were primarily observed in junctures between epidermal cells, however, very often bacteria could be seen in the central part of the root and also in vessels.

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МОДЕЛЮВАННЯ МОЛЕКУЛЯРНИХ ВЗАЄМОДІЙ БАКТЕРІЙ З РОСЛИНАМИ ДЛЯ ПОЛІТНИХ ЕКСПЕРИМЕНТІВ

Н. О. Козировська, Г. Л. Ковтунович, О. В. Лар, М. В. Ковальчук, В. В. Негруцька, О. С. Корнійчук, І. С. Рогуцький, А. П. Алпатов, В. А. Кордюм

Вирощування рослин у космічних відновлювальних оранжереях є актуальним для підтримки систем життєзабезпечення космонавтів у тривалих польотах. Однак відомо, що космічні фактори впливають на експресію генів, що регулюються системами, чутливими до цих факторів. На борту космічних апаратів ризик генетичних перебудов підвищується, тому слід очікувати деяких змін у ДНК. Як наслідок, бактерії можуть проявляти нові властивості, наприклад фітопатогенність. З попереднього досвіду ми знаємо, що бактерії посилюють внутрішню колонізацію коренів рису під час космічного польоту. Для розуміння цих даних, а також передбачення підвищеної агресивності бактерій по відношенню до рослини-господаря необхідно на моделях вивчати молекулярно-генетичні взаємодії рослини з бактеріями, які відбуваються під впливом фізичних факторів. Гени, що кодують бактеріальні пектинази, є зручною моделлю для вирішення взаємопов'язаних завдань стосовно взаємодій рослини з бактеріями.