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Modeling the pNARSLux transfer in the wheat rhizosphere under simulated microgravity

Model experiments on conjugal plasmid transfer between bacteria in the wheat rhizosphere under simulated microgravity (SMG) were performed to evaluate potential of natural gene flow and risk of genetic recombinations in microorganisms in space gardens under stressed conditions of space flight. Strains of bacteria *Klebsiella oxytoca* IMBG 26, *Pseudomonas* sp. 7, *P. putida* 9, *Agrobacterium tumefaciens* 9023, *Escherichia coli* S-17 were selected for matings in the wheat microcosm on a clinostate with horizontal mode of rotations within a ten-day period. Transfer of pNARSLux was not detected in both clinorotated and control variants in sterile conditions. The changed vector of gravity influenced both plant development, resulted in a shorter seedling heights, and in bacteria spread on the surface of wheat roots resulted in a less amount of cells. In both variants of experiments (normal gravity and SMG) positive impact of bacteria on wheat seedling development is displayed as compared with control noninoculated plants.

INTRODUCTION

Space flight exposes human beings to physiological and psychological health risks from radiation, reduced gravity, and isolation. One of the means that will reduce psycho-emotional loading to astronauts in space and planetary environments is a space garden. As far the garden is highly populated by microorganisms, it is a potential environment for a gene transfer, DNA recombination, and for a selection of organisms with novel characteristics. This should be encountered during manned space flight and invested more effort in investigation of impact of cosmic stress factors on behavior of microorganisms.

Conjugative transfer of bacterial plasmids is the most efficient way of horizontal gene spread, and it is therefore considered one of the major reasons for study of bacteria in space gardens. Horizontal gene transfer, the intraspecies and interspecies exchange of genetic information, plays an important role in accommodation of bacteria enabling them to rapidly respond to environmental challenges [2]. The most important contributor to horizontal gene transfer is the heterogeneous group of mobile genetic elements that includes plasmids, insertion elements, transposons, integrons, phages, and genomic islands [1, 12, 21].

Conjugation is one of the three major mechanisms (along with transformation and transduction), that provide bacterial populations with access to a "horizontal gene pool". It should be taken into account that the barrier of horizontal gene transfer may be eliminated in the space environment. Plasticity of bacterial genome may create novel recombinant plasmids or other genetic elements by means of *in vivo* successive transpositions of elements from chromosome, and it may result in recombination processes and formation of organisms with unpredicted traits [18]. Mobilizing or retromobilizing plasmids present in indigenous soil bacteria could potentially still effect the transfer of the less mobile heterologous DNA via chromosome or plasmid mobilization, which may involve cointegration [6]. Such plasmids might thus be responsible for the escape of the DNA elements from bacteria introduced into soil. Our experience shows that acquisition of plasmids by pathogenic bacteria led to an increase of their aggressiveness [9]. We may postulate that risk of GMO formation and than colonization of appropriate niche without selective pressure might be higher in space flight than on Earth.

Gene transfer in soil via conjugation has received much attention, and the focus of most studies has been the transfer and fate of introduced plasmids [5,

Table 1. Stability of the Lux⁺ plasmids in *Klebsiella oxytoca* IMBG 26

Strain	Percentage of the stable clones after n generations			
	10	20	50	100
pVG37Lux*	12	2	< 1	—
pKAS18Lux*	86	78	34	1
pBR322Lux*	3	< 1	—	—
pNARSLux	100	100	100	98

* Plasmids from laboratory collection.

15, 17, 18, 20, 26]. It was assumed for a long time that gene transfer between different species of microorganisms is a very rare event; later, however, it was shown that under favorable conditions, in specific soil microhabitats, or under selection conditions, both self-transmissible and mobilizable plasmids present in introduced hosts can be transferred to introduced recipients, as well as to a variety of indigenous bacteria [11, 14, 23] in different microhabitats, including plant tissue [9], and even human cells [4]. To our knowledge, rhizospheres of plants also provide conditions conducive to conjugal plasmid transfer between bacterial inhabitants [11, 23, 25].

There is a paucity of knowledge concerning the effects of cosmic factors on plasmid prevalence and transfer [7]. Stresses, resulting from microgravity and other cosmic factors, may enhance plasmid transfer between bacteria, whereas it has been suggested that chemical stress has been found to exert an enhancing effect [8]. In this report we describe experiments aiming to shed a light on conjugation that may take place in bacterial populations colonized the plant in under simulated microgravity.

Evaluation of a pattern of bacteria colonization. Bioluminescence method was used for evaluation of colonization and survival of bacteria in the rhizosphere when a colonization pattern of the plant in microcosms can be visualized by naked eyes in darkness. pNARSLux served for this purpose [10]. As experiments showed the plasmid was stable within many generations of bacteria (Table 1), and it could be used in long-term experiments. Data of experiments on planting of inoculated seeds of wheat (*Triticum aestivum* var. Katyusha) and mustard (*Brassica arvensis*) into different substrates exhibited a full-length colonization of roots of both plants by the Lux⁺ *K. oxytoca* within 10–12 days (mineral substrates) or 8 days (the agar medium). Another variant of inoculation was an injection of bacterial suspension into a spot near untreated 7-day old roots through a window in a cultivation chamber (CC). Roots of all seedlings grown in the agar substrate were colonized in full after 7 days (Figure).

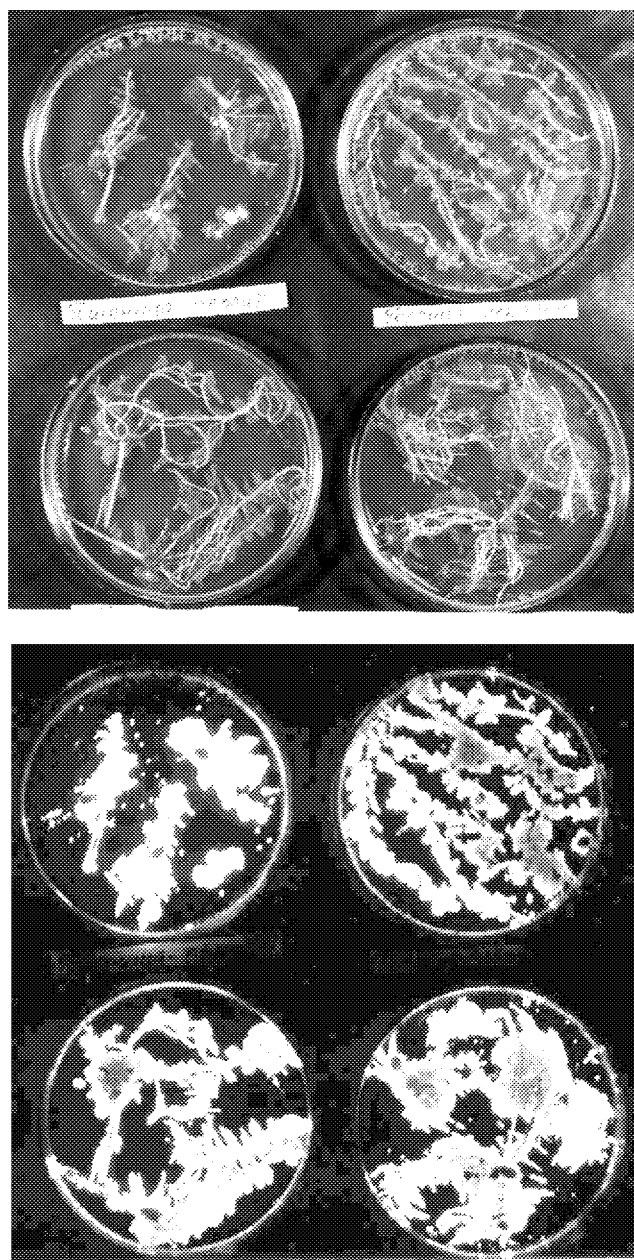


Figure. Bioluminescence of the marked bacterium *K. oxytoca* VN13 (pNARSLux) on roots of the plants. On top — in a light; below — in a darkness

Conjugal transfer of replicative plasmids. Experiments were performed in a microcosm that allowed us to work under sterile conditions. Polypropylene containers (200 by 55 mm) were used to produce microcosms. The microcosms were planted with three pregerminated wheat seeds. They were incubated for 10 days in a climate chamber with a light-dark cycle

Table 2. Transfer of recombinant plasmids from *E. coli* to Gram-negative bacteria *in vitro* experiments

Plasmid	Recipient	Frequency of transfer
pSUP2021	<i>K. oxytoca</i>	$2.0 \cdot 10^{-3}$
	<i>Pseudomonas</i> sp.	$2.0 \cdot 10^{-7}$
	<i>P. putida</i>	$2.0 \cdot 10^{-7}$
	<i>A. tumefaciens</i> .	$1.0 \cdot 10^{-5}$
pNARS	<i>K. oxytoca</i>	$2.0 \cdot 10^{-3}$
	<i>Pseudomonas</i> sp.	$0.5 \cdot 10^{-7}$
	<i>P. putida</i>	$2.0 \cdot 10^{-6}$
pNARSLux	<i>A. tumefaciens</i> .	$9.0 \cdot 10^{-6}$
	<i>K. oxytoca</i>	$2.0 \cdot 10^{-4}$
	<i>P. putida</i>	$1.0 \cdot 10^{-7}$
pCAM120	<i>A. tumefaciens</i> .	$7.0 \cdot 10^{-6}$
	<i>K. oxytoca</i>	$5.0 \cdot 10^{-6}$
	<i>Pseudomonas</i> sp.	$0.5 \cdot 10^{-6}$
	<i>K. oxytoca</i>	$1.0 \cdot 10^{-6}$

of 16 and 8 h at 20°C in different substrates. The natural conjugal gene flow in gram-negative bacterial pairs in wheat rhizosphere in different substrates of microcosm has been studied in normal gravity: a) an agar medium; b) sterile lecatone; c) nonsterile lecaton and zeolite; d) nonsterile lecaton and peat. Clinorotations of microcosms have been performed at Kyiv State University in horizontal mode during 10 days, using universal clinostate «Cycle-2».

Plasmids pSUP2021 [16], pNARS, and pNARSLux [10] were used in microcosm experiments. For mating experiments the donors, *E. coli*, harbored plasmid which conferred resistance to antibiotics and the recipients that had chromosomal rifampicin resistance marker (*P. putida*, *Pseudomonas* sp. 7, *A. tumefaciens* 9023, *K. oxytoca* IMBG 26) were used. Thus, transconjugants could be enumerated by isolating from roots and plating on selective medium, containing two antibiotics directed against both donors and recipients, as well separately donor and recipient have been enumerated on plates with appropriate antibiotics.

On agar plates transfer of plasmids pSUP2021, pNARS, and pNARSLux from *E. coli* strains to selected bacteria varied at frequencies from $2.0 \cdot 10^{-3}$ to $2.0 \cdot 10^{-7}$ transconjugant per recipient when experiments were performed in selective conditions (in presence of antibiotics used against parents) (Table 2).

In the wheat rhizosphere plasmid transfer has been examined between donor *E. coli* S-17 and recipients *P. putida*, *Pseudomonas* sp. 7, *A. tumefaciens* 9023, *K. oxytoca* IMBG 26. In microcosms we tried to detect

natural gene flow, without selective pressure. Wheat seeds were surface sterilized, germinated within 2 days, and inoculated with suspension of donor first and recipient a day later (both 10^7 cfu/ml). Procedure of isolating of putative transconjugants was following: 100 mg roots were minced in aseptic conditions, a crushed tissue diluted and spread on selective agar plates. In variant *P. putida* — *E. coli* S-17 (pNARS lux) (agar or lecaton substrates) a plasmid transfer was very low (less 10^{-7}). Between pairs of *E. coli* — *A. tumefaciens* 9023, *E. coli* — *K. oxytoca* IMBG 26, and *E. coli* — *Pseudomonas* sp. 7 transconjugates were not revealed in either case.

Exchange of genetic material between *E. coli* S-17 (pSup2021) and *Pseudomonas* sp. 7 was modeled in variant when bacteria were introduced into the rhizosphere separately in a 2 day interval. In such a variant transconjugants were not detected, even after a supplement of glucose (2 %, final concentration) into substrates with aim to enhance multiplication of bacteria.

There were conditions for matings between bacteria of microcosm in nonsterile conditions when mixtures lecaton — zeolite and lecaton — peat were used as substrates, however, matings occurred between donors *E. coli* and indigenous bacteria originated from substrates.

Exchange of genetic material between *E. coli* S-17 (pNASLux) and *Pseudomonas* sp. 7 and *E. coli* S-17 (pNASLux) and *K. oxytoca* IMBG 26 in the rhizosphere of wheat grown in agar substrate was modeled under conditions of simulated microgravity (SMG). SMG is generated in a clinostate. By rotating at constant velocity around horizontal axis an environment is produced in which the gravitational vectors are randomized over the surface of the plant, resulting in an overall-time-averaged gravitational vector. Wheat seeds were surface sterilized, germinated within 2 days, and inoculated in two variants: 1) first — with donor cell; 2) first with recipient cells. After 2 days second partner was used for inoculation, respectively. Controls were kept at normal gravity. Clinorotations were performed during 10 days. Results of a transconjugant search were not successive (Table 2), that may mean that horizontal rotation either does not favour a mating between partners or donors and recipients overgrow newly formed transconjugants, and latter, being less competitive, do not survive (1); there is no quorum (not enough bacterial cells) for making contacts in microhabitats as a consequence of deregulation of a quorum sensing under SMG (2) [3].

Conjugal transfer of suicide plasmid. Transposon migration has been simulated among bacteria of

Table 3. Matings of bacteria in the wheat rhizosphere under simulated microgravity

Variant	Transconjugants Km ^r , Rif ^r , cfu/g	Donor, Km ^r cfu/g	Recipient, Rif ^r cfu/g
<i>E. coli</i> — <i>Pseudomonas</i>			
Control	0	4.6 ± 1.5 · 10 ⁵	2.4 ± 3.6 · 10 ⁷
Clinorotation	0	1.1 ± 5.3 · 10 ⁴	8.0 ± 7.0 · 10 ⁶
<i>E. coli</i> — <i>Klebsiella</i>			
Control	0	6, 2 ± 2.1 · 10 ⁵	2.4 ± 2.0 · 10 ⁸
Clinorotation	0	1.4 ± 3.3 · 10 ⁴	1.5 ± 5.4 · 10 ⁸

Table 4. Height of the wheat shoots under simulated microgravity

Variant of microcosm	Height of shoots, mm
Inoculated Wheat	21.0 ± 0.2
Noninoculated wheat	20.0 ± 0.2
Inoculated Wheat (clinorotation)	16.3 ± 0.3
Noninoculated wheat (clinorotation)	15.0 ± 0.2

rhizosphere population, using a suicide vector that has been mobilized from a donor cell of bacterium to a recipient one. Transposon mTn5SsgusA20 which confers a *Paph-gusA-trp* cassette, containing reporter the *gusA* (beta -glucuronidase) gene, was inserted into suicide vector pCAM120 based on pUT/mini-Tn5 and introduced to a mobilized strain of *E. coli* [27]. Two-parental mating between *E. coli*, conferring transposon, and two strains of bacteria — *K. oxytoca* and *P. fluorescens* has been simulated in on plates at first. Frequency of transposition of mTn5SsgusA20 was evaluated in recipient strains as 10⁻⁶ cfu/ml. Two-parental matings between *E. coli* (pCAM120) and *Pseudomonas* sp. 7 have been performed in the wheat rhizosphere (lecaton substrate placed into CC). A frequency of transposition of mTn5SsgusA20 was 6.0 · 10⁻⁶ transposant/donor · g. Under conditions of SMG transposants were not detected.

Impact of simulated microgravity on microcosm organisms. Two different results exhibited impact of SMG on microcosm organisms. 1) A wheat growth of rotated variants was smaller (Table 3). Height of shoots, length of roots, and biomass of 12 day-old wheat seedlings under clinorotation were less than in control variants. This is in agreement with data of L. T. Mischenko that produced wheat microcosms in SMG [13].

2) Total number of bacteria isolated from the wheat root in rotated variants was smaller (per gram) (Table 2, 4). Survival of donor cells in the wheat rhizosphere under SMG appears to be more poor than

Table 5. Number of *Klebsiellaoxytoca* IMBG 26 cells isolated from the wheat root under simulated microgravity

Variant	<i>K. oxytoca</i> , cfu/g	<i>E. coli</i> , cfu/g
Inside roots	5.0 · 10 ⁶	4.4 · 10 ⁴
On the surface	2.4 · 10 ⁸	4.1 · 10 ⁵
Inside roots (clinorotation)	1.0 · 10 ⁶	1.0 · 10 ⁴
On the surface (clinorotation)	1.5 · 10 ⁷	1.7 · 10 ⁴

in normal gravity, and data coincide with earlier results got by Ukrainian researchers under a leadership of V. A. Kordyum from a series of flight experiments [22].

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МОДЕЛЮВАННЯ ПЕРЕМІЩЕННЯ ПЛАЗМИДИ pNARSLux У РИЗОСФЕРІ ПШЕНИЦІ ЗА УМОВ ІМІТОВАНОЇ МІКРОГРАВІТАЦІЇ

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У контексті необхідності передбачення переміщення генів між бактеріями та оцінки ризику виникнення рекомбінаційних подій у мікроорганізмів у космічній оранжереї під час стресових умов космічних польотів проведено низку модельних експериментів з переносу кон'югативної плазмиди між бактеріями у ризосфері пшениці. Штами бактерій *Klebsiella oxytoca* VN13, *Pseudomonas* sp. 7, *Agrobacterium* sp., *Escherichia coli* S-17 було обрано для моделювання переміщення генетичного матеріалу між бактеріями у мікрокосмі пшениці за умов імітованої мікрогравітації (ІМГ), яку створювали на клиностаці при горизонтальному обертанні мікрокосму навколо осі протягом 10 діб. У жодному з варіантів не знайдено переносу плазмиди pNARSLux бактеріями у стерильних умовах. Проте дія ІМГ виявлялася у повільнішому розвитку рослин та зменшенні кількості бактерій на поверхні коріння пшениці. В обох варіантах дослідів (ІМГ та нормальна гравітація) простежувався позитивний вплив бактерій на розвиток рослин у порівнянні з контролем рослин без введення бактерій.