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BIOLUMINESCENCE-BASED DETECTION OF KLEBSIELLA OXYTOCA VN13 IN THE ENVIRONMENT

The continuous expression of the Photobacterium leiognathi 54D10 lux genes coding for the bioluminescence was obtained in Klebsiella oxytoca VN13. Chromosomally and plasmid-encoded bioluminescence of strains constructed was used to monitor their survival in the barley rhizosphere during a limited field introduction in parallel with the parental strain. Lux$^+$ Derivatives of K. oxytoca VN13, carrying the bioluminescence reporter plasmids survived on roots during the whole vegetative period of the plant and were not isolated from soil.

Introduction. Monitoring of bacteria in the environment accompanies a problem of bacteria releases. The efficiency of detection is critical in tracking the bacteria fate in natural biocenosis. At present rapid and sensitive molecular biological methods to detect bacteria in the environment have largely replaced conventional plating methods (see review [1]). New promising techniques based on recognition of the species/strain specific DNA sequences have been developed recently, and might be used to monitor bacteria in environmental samples [2—5]. The DNA probing technology enhanced by the polymerase chain reaction is sensitive and may be used for detection of nonculturable bacteria or bacteria present at low density in the microbial community [6]. However, results gained from probing analyses give information about events that took place in the past. Equally limiting is the destructive nature of new methods and their high cost.

Another approach to detection of bacteria in the environment is using of genetic markers as a tagging system for bacteria identification in a natural community. The *lux* genes possess the advantage over the reporter markers, such as *lacZY*, *xylE*, or *gusA*, because bacteria engineered to bioluminescence can be monitored during ongoing process and in nondisruptive manner. The light emission of bioluminescent bacteria can be measured rapidly and at a little cost. The sensitivity of bioluminescence measurements is comparable to detection by hybridization technique [1].

A variety of bacterial hosts have got a luminescent phenotype after introduction of the complete *lux* operon, and their bioluminescence has been used to monitor the process of plant-bacterial interaction [7]; to quantitate the number of specific bacteria [8, 9]; to assess the survival of bacteria in the rhizosphere in environmental simulations [10]; to observe persistence and movement of bacteria during a limited field introduction [11]. The use of the *lux* genes as the marker system for long-term bacteria monitoring in the environment was discussed as problematic because of the energy-consuming expression of the *lux* genes had to reduce the survival of the target cells [1]. This study was undertaken for long-term monitoring of the survival of *K. oxytoca* VN13 in new natural surrounding by both traditional plating and bioluminescence-based methods.

Materials and methods. Strains and plasmids. Strains and plasmids used in this study are listed in Table 1. All microplot

tests were performed with *K. oxytoca* VN13 and its derivatives. *K. oxytoca* VN13 was isolated from the interior of a rice root taken from a Vietnamese rice paddy [12]. The set of *lux* genes of *Photobacterium leiognathi* used to tag *K. oxytoca* VN13 was derived from *pVG37Lux*, kindly provided by Vladimir Gurevich (Krasnojarsk Institute of Biophysics of RAN, Russian Federation) [13]. The set of seven *lux* genes (*luxA* and *luxB* are structural genes of the luciferase biosynthesis, *luxC*, *luxD*, and *luxE* encode the synthesis and recycling of aldehyde substrate, *luxF* and *luxG* are of unknown function) was cloned in *pVG37Lux* (personal communication).

Two nonconjugal plasmids *(pKAS18 and pMAK705)* based on different replicons (*pZE8* from *Citrobacter freundii* and *pSC101*, respectively) were used to subclone the *lux* genes from *pVG37Lux*. *pKAS18Lux* was constructed by inserting the *lux* genes taken as a *BamHI* fragment from *pVG37Lux* into the *BamHI* site of *pKAS18* polylinker. The same strategy was used for construction of *pMAK705Lux*. *pRT733Lux* was constructed by inserting the *lux* genes taken as a *BamHI* fragment from *pVG37Lux* into the *BglII* site of *IS50R* of TnphoA of *pRT733* and transformed into *Escherichia coli* SM10 lambda *pir*, because *pRT733* and its derivative require *pir* in the chromosome of that strain for replication. *pRT733Lux* was used as a suicide vector to deliver *TnphoALux* to *K. oxytoca VN13*. The plasmid was transferred from *E. coli* to *K. oxytoca* by conjugation as described [14]. Exconjugants were selected as exhibiting resistance to kanamycin (conferred by *TnphoALux*) and to rifampicin (conferred by the chromosome of *K. oxytoca* VN13). Plasmid isolation and gene cloning procedure were performed by standard methods [15]. *K. oxytoca* VN13 was transformed by the method of Alexeyev, Gun'kovskaya [16]. The stabilities in *K. oxytoca* VN13 of plasmids carrying the *lux* genes were determined by plating on nonselective medium aliquots of cultures grown up to 100 generations in the absence of selection. At least 500 colonies were screened for each aliquot.

K. oxytoca VN13 strains were grown in Luria broth and on LB agar [14]. When appropriate, ampicillin or chloramphenicol were added to a concentration of 50 μg/ml and kanamycin or rifampicin to a concentration of 100 μg/ml. Bacteria were grown at 37 °C.

Seed inoculation and germination. An overnight liquid culture of *K. oxytoca* VN13 was washed and diluted to a concentration of 10^8 cfu/ml prior to inoculation of barley seeds. For laboratory experiments seeds were sterilized with chloramine b (1 %). Medium [17], deficient in carbon and nitrogen sources, was used to germinate seeds. Inoculated seedlings were grown at room temperature and natural light. Randomly situated microplots (1×1 m) were used in two replications for either type of bacteria tested.

Table 1
Bacterial strains and plasmids

Strain or plasmid	Phenotype or genotype	Source or reference
Strains		
K. oxytoca VN13	Rf^r	Laboratory collection
E. coly SM10	lambda *pir*	Taylor et al. [22]
Plasmids		
pVG37Lux	Ap^r *Lux*	V. Gurevich
pKAS18	Km^r ori *pZEB*	Alexeyev et al. [23]
pKAS18Lux	Km^r ori *pZEB Lux*	This study
pMAK705	Cm^r repts *pSC101*	Hamilton et al. [24]
pMAK705Lux	Cm^r repts *pSC101Lux*	This study
pRT733	Ap^rKm^r *TnphoA*	Taylor et al. [22]
pRT733Lux	Ap^rKm *TnphoALux*	This study

Identification of bacteria reisolated from barley. The roots of barley inoculated with the parental strain of *K. oxytoca* VN13 were washed 1 hr (with shaking) with a 0.9 % solution of sodium chloride supplemented with 0.1 % tween-20, and the extract was plated on selective agar medium. Analysis was performed monthly using 3 samples of the inoculated barley. The identity of antibiotic-resistant bacteria as *K. oxytoca* was confirmed by fingerprinting of total proteins, as described by [18].

Bioluminescence detection. Light emission by strains of *E. coli* and *K. oxytoca* bearing the *lux* operon was detected visually in a dark room. Light emission from roots was detected by autophotography or by visual inspection after enrichment of bacteria on selective medium. Soil was removed from the roots of seedlings, and the roots were sealed in plastic bags and exposed to RM-V X-ray film («Svema», Ukraine). The films were developed as prescribed by the manufacturer.

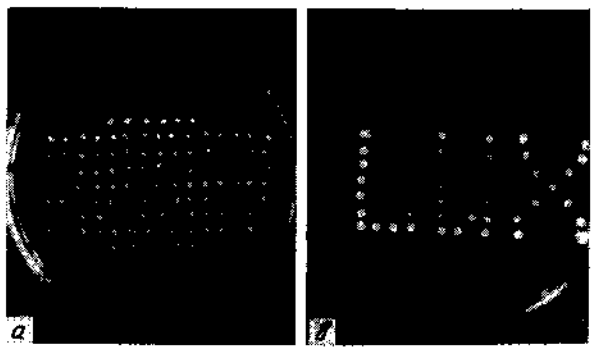

Fig. 1. The colonies of the parental *K. oxytoca* VN13 strain and the luminescent *K. oxytoca* VN13 (*pKAS18Lux*) strain: light photography (*a*), photography made in a dark room (*b*)

For a series of large-scale analyses of the barley roots sterile plastic transparent boxes with a special lid (d=25 mm) filled with selective agar were used (Fig. 2, *a*, *b*). Different parts of the plant roots were placed on agar inside boxes brought to a field, and samples were incubated at 30 °C overnight. Bioluminescent bacteria grown on selective agar were identified by inspection of the lids in a dark room. Detection of bioluminescence was performed every two weeks using 20 randomly selected barley plants for each type during May-September, 1992 and after a year, in April, 1993. Soil samples were collected for analysis in the rhizosphere of inoculated plants and between rows of barley. 100 mg of soil sample was minced, diluted, and plated on selective agar.

RESULTS. Construction of K. oxytoca strains with Lux+ phenotype. It was preferable to insert the *lux* genes into chromosome of *K. oxytoca* VN13 where their stability might be increased in case of nonselective conditions in limited field experiment. We inserted the *lux* genes into the chromosome by transposon mutagenesis. *TnphoLux* was transferred into *K. oxytoca* VN13 by conjugation, as described in Materials and Methods. A low level of bioluminescence in recombinant bacterium was obtained, and it would be difficult to detect it visually on the root system. In further experiments two new Lux+ derivatives of *K. oxytoca* VN13 bearing *pKAS18Lux* or *pMAK705Lux* were constructed, and they were expected to be bright enough because of a *lux* genes high dosage. The plasmid-bearing strains exhibited a high level of bioluminescence (Fig. 1). Light emission from colonies of *K. oxytoca* VN13 *(pKAS18Lux)* and *K. oxytoca* VN13 *(pMAK705Lux)* could be observed immediately after placing plates in a dark room. Since under field conditions plasmid-bearing *K. oxytoca* VN13 will not be subjected to antibiotic selection, it was important to ascertain the stability of both *pKAS18Lux* and *pMAK705Lux* within *K. oxytoca* VN13 in the absence of selection. Results showed that while *pKAS18* and *pMAK705* are maintained without selection for at least 100 generations, their Lux+ derivatives are much less stable. In particular, the level of the *lux* plasmids in the population decreased to undetectable levels after 100 generations of unselected growth.

Detection of the tested bacteria in the rhizosphere. In laboratory experiments sterilized barley seeds were inoculated with the recombinant luminous *K. oxytoca* VN13 *(pKAS18Lux)* and *Lux*+ transposant separately and placed on minimal agar in tubes. Bioluminescence was detected on the barley seedling roots in a dark room by unaided eyes, and it was concluded that the genetically modified *K.*

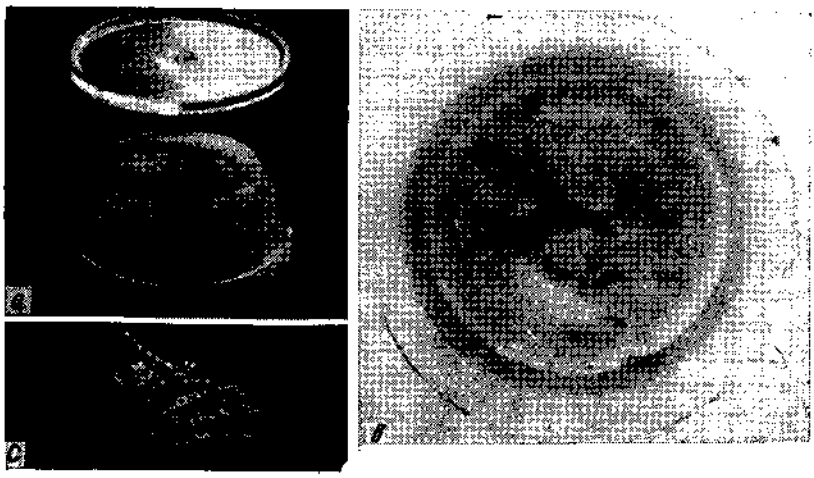

Fig. 2. A plastic transparent box used for enrichment of bioluminescent bacteria (*a*); a fragment of the barley root placed on selective agar inside a box lid (*b*), bioluminescence of bacteria localized on a fragment of 3.5-month old barley root and selectively enriched inside a box (exposure 20 min) (*c*)

oxytoca VN13 could colonize efficiently the root system of this plant. Bacteria colonizing the barley roots were reisolated on nutrient medium after 14 day since inoculation, screened on light emission, and transferred on medium supplemented with kanamycin in order to study both plasmid and transposon stability. Reisolated bacteria revealed unstability and gave 12 % Lux+Km^r (transposant) and 59 % Lux+Km^r forms *(pKAS18Lux)*.

For a field microplot test we used three variants of the recombinant *K. oxytoca* VN13 marked with the *lux* genes and the parental strain. Bioluminescence detection in the rhizosphere of barley seedlings by autophotography was performed after the first 14 days since inoculation. Autophotographs of root infected by *Lux*+ transposant were obtained only after addition of nutrient medium to bags containing roots prepared for exposure to film. The plasmid-containing Klebsiella strains showed the pictures appeared on developed films after 2 hr exposure without addition of nutrients. Autophotographs reflected the distribution of the luminescent bacteria along the root (data not shown). Later we used cheaper technique than autophotography which was an enrichment of bacteria on selective agar in boxes followed by visualization of bacterial bioluminescence in darkness. The roots colonized by *Lux*+ transposant gave weak visible light signals on selective medium in boxes, and after 6 weeks of inoculation it was not detected (Table 2). *Lux*+ derivatives of *K. oxytoca* VN13 bearing *pKAS18Lux* or *pMAKLux* showed a surprising survival in the barley rhizosphere during the spring-autumn period of the barley vegetation. Bioluminescent bacteria were distributed along the barley root system, as reflected Fig. 2, *c*. Only after harvest the population of *Lux*+ bacteria colonizing plants diminished, and a number of plants colonized by bioluminescent bacteria decreased more than 2-fold in September (see Table 2). Similar results showed the study on the survival of the *K. oxytoca* VN13 parental strain. Attempts to detect *K. oxytoca* VN13 of both marked and original strains in soil samples were unsuccessful. Bacteria tested were not detected in April, 1993.

Discussion. We met a problem of bacteria monitoring in the environment because of *K. oxytoca* VN13 introduction into new climate zone. Being isolated in Vietnam from the rice root along with two accompanied bacteria, *K. oxytoca* VN13 exhibited beneficial effect on different plants in green-house trials (unpublished data). This bacterium possesses the advantage over rhizosphere bacteria: the capability to colonize the plant root interior and use that ecological niche for recolonization of the plant surface. The stable introduction of microbes of a beneficial nature into the rhizosphere of plants has proven to be difficult because they are usually outcompeted by the indigenous microflora [19]. We studied the survival of *K. oxytoca* VN13, bacterium with expected beneficial effect on the plant, during limited introduction of it to a new climate zone. The presence of bacterium on the barley roots was monitored by a conventional sampling method, and it was used in parallel with a bioluminescent technique in order to prove its usefullness for long-term monitoring of bacteria in the environment. The *lux* genes from marine bacterium *Ph. leiognathi* were used to construct strains of *K. oxytoca* VN13 with the luminous phenotype, and tagged bacteria were observed during period of the plant-host vegetation by means of light emitted.

Application of the *lux* genes for detection of bacteria in the environmental samples normally requires the availability of instrumentation for the visualization and quantification of photon emission such as fiber optics, photoncounting electronic equipment or the autophotography technique [20]. Using these devices, information on the survival, quantity, and sites of preferential localization can be gained in a non-disruptive manner. For the initial screening of tested bacteria in the rhizosphere it is enough to receive information whether bacteria are present in its ecological niche or not, and to evaluate approximately a rate of the root colonization. In this study we did not use devices for detection of bioluminescent bacteria on the plant roots but the technique of visual inspection of enriched bacteria present on the root samples. This technique requires an availability of the *Ph. leiognathi lux* genes involved in synthesis of both luciferase and its substrate, selective agar inside small boxes, a dark room, and unaided eyes. Since it is inexpensive and nonlabour-intensive, it offers the advantage to screen quickly hundreds of samples under field conditions. It can be used for initial selection of the environmental samples in field experiments and adapted to laboratories with a low level of instrumentation.

Using the technique of visual inspection of bioluminescent bacteria enriched on selective medium we revealed that plasmid-bearing variants of *K. oxytoca* VN13 exhibited stable presence on the plant root from May to September, 1992. *Lux*+ transposant had a bit poorer survival but it was observed in the barley rhizosphere during 6 weeks, and it is similar

Table 2

Survival of the genetically engineered K. oxytoca VN13 in the barley rhizosphere (a field microplot test)

Strain	Number of plants colonized by *K. oxytoca* VN13 strains										
	1992									1993	Total number of plants screened
	May		June		July		August		September	April	
	11	25	15	29	13	27	10	24	4	26	
pKAS18Lux	20	20	20	20	19	15	16	9	8	0	20
pMAK706Lux	20	20	20	20	18	18	16	11	0	0	20
Tnpho-lux	7	1	0	ND	ND	ND	ND	ND	ND	ND	20
Parental	3	ND	3	ND	3	ND	3	ND	1	0	3

ND—not determined.

to the result obtained by J. Shaw group earler [11]. The reason of poor ability to survive of *K. oxytoca* VN13 with chromosomally encoded bioluminescence might be explained by a transposase-mediated rearrangements that led to unstability of the *lux* marker. By comparing the survival of the parental strain with the *lux* plasmid-bearing ones during the plant vegetation period, we may conclude that extra genes expressed did not decrease the survival of the engineered strains in the rhizosphere.

The finding that tested plasmid-bearing bacteria continuously expressed the *lux* genes and survived on the barley root system during a long-term period was unexpected for us because the *lux* marker was unstable in continuous culture. Earlier plasmid encoded bioluminescence was employed for detection of bacteria in the plant rhizosphere in microcosm experiments [7, 10]. Stable expression of the *Vibrio fisheri lux* CDABE genes was detected by J. Shaw and C. Kado in the phytopathogenic bacterium *Xanthomonas campestris pv. campestris* 2D520 in infected cauliflower plant. Another results provided by group of L. de Weger demonstrated poor survival on soybean roots of *Pseudomonas fluorescens* WCS374 cells containing constitutive bioluminescence plasmid, and it was concluded by these authors that the low bioluminescence activity of cells in the rhizosphere was reduced because of a high energy demand to synthesize the aldehyde continuously. Data concerning the bioluminescence energetics prove that luciferase does not consume ATP for oxydation of aldehyde but reduced flavin mononucleotide ($FMNH_2$), and it has to decrease a level of generation of ATP from electron transport system in cell considerably. Furthermore, fatty acid reductase does demand stochiometric quantity of ATP for production of 1 mol of aldehyde [21]. Taking into account these data and evaluating results obtained by three different groups exploited interacted with plants bacteria we may suppose that the available plant host has to play a selective role in the survival of bioluminescent bacteria, and latter, being in a close contact with the plant may be provided with energy from the host. On the contrary, the survival ability in soil of bacteria tagged with the *lux* genes was found to be less than that in the rhizosphere [8, 11].

Our results show that the endorhizosphere bacterium *K. oxytoca* VN13 survives and it is an active in the rhizosphere during the whole period of the host-plant vegetation. Competition of the genetically engineered klebsiella against microbes from a natural community is not too dramatic, as expected, because of a specific ecological niche. Further experiments performed on the same model system have to show a selective role of the plant in the survival of endorhizosphere bacteria.

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ВИЯВЛЕННЯ БАКТЕРІЙ KLEBSIELLA OXYTOCA VN13
У НАВКОЛИШНЬОМУ СЕРЕДОВИЩІ МЕТОДОМ БІОЛЮМІНЕСЦЕНЦІЇ

Р е з ю м е

Конститутивну експресію *lux*-генів *Photobacterium leiognathi* 54D10, що кодують біолюмінесценцію, отримано в бактерії *Klebsiella oxytoca* VN13. Для спостерігання за виживанням бактерій у ризосфері ячменю в польових експериментах було сконструйовано штами з закодованою в хромосомі або плазміді біолюмінесценцією. Похідні *K. oxytoca* VN13, які містили плазміди з біолюмінесцентною ознакою, виживали на коренях ячменю протягом усього періоду вегетації рослини, але їх не було виявлено в грунті.

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