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Statement of the ZKBS on the risk assessment of the ARSOlux test system

General information

The ARSOlux test system has been developed by the Helmholtz Centre for Environmental Research (UFZ, Leipzig) in order to determine the arsenic concentration in water samples. It is based on genetically modified bacteria which are derived from the laboratory strain K12 of *Escherichia coli* and which emit light after coming in contact with arsenic compounds.

The UFZ has made an application in Mongolia for conducting a study to determine the arsenic content in groundwater. The competent authority, the Mongolian National Biosafety Committee (NBC), thereupon requested from the ZKBS (Central Committee on Biological Safety, Germany) a risk assessment of the genetically modified organisms used pursuant to Article 15 in conjunction with Annex III of the Cartagena Protocol. The objective of such risk assessment (see item 1 of Annex III) is "…to identify and evaluate the potential adverse effects of living modified organisms on the conservation and sustainable use of biological diversity in the likely potential receiving environment, taking also into account risks to human health… " [1].

Description of GMOs used

The *E. coli* strain used in the ARSOlux system is a derivative of the laboratory strain K12, into which a plasmid was transferred. This strain is referred to as *E. coli* DH5α-2697.

Recipient organism

E. coli belongs to the family of *Enterobacteriaceae*. This family comprises Gram-negative, facultatively anaerobic, non-sporulating rods, which make up 0.1% of the intestinal flora of homeotherms. Some pathogenic types can induce severe human diseases.

K12 strains are derived from an isolate found in the stool of a convalescent diphtheria patient from 1922 [2]. Widely used for bacteriological studies since the 1940s, this isolate constitutes a very well-characterised apathogenic organism which is also today widely used as a model organism in research and industry. Its apathogenicity is explained by the fact that the virulence factors typical of pathogenic strains are not expressed by the K12 strain and its derivatives. Due to defects in the bacterial cell structure, the colonisation of human and animal intestines is not possible under normal conditions. For example, the lipopolysaccharide core structure in the outer membrane is changed, so that the adhesion of O antigen polysaccharide side chains is disturbed [3]. This is accompanied by a changed composition of the glycocalyx surrounding the bacteria, preventing adhesion to the mucosa of the intestinal epithelium [4]. Furthermore, K12 strains express no adhesins and capsular antigens [3]. The survivability of K12 strains in intestines has been tested in humans and animals in a multitude of studies. According to these studies, the intestinal survival period of the bacteria is between 1 and 6 days [5-8].

In addition to their survivability in intestines, pathogenic *E. coli* strains are characterised by their pathogenicity determinants. Besides the pathogenic determinants mentioned above, others include e.g. toxins, invasins and aerobactins [9]. The corresponding genes are not present or not expressed in the genome of *E. coli* K12 strains. Consequently, *E. coli* K12 strains are not assumed to pose a risk to humans and animals when inhaled or ingested.

The natural habitat of *E. coli* is the intestinal tract of mammals. Due to the above-mentioned genomic changes, *E. coli* K12 and its derivatives are not able to colonise the intestines or survive therein. Furthermore, numerous studies with water and soil samples indicate that these strains cannot survive in the environment either. In river water the number of viable *E. coli* K12 cells decreases from 3 x 10^6 cells/ml to approx. 10 cells/ml within 6 days [10]. Other tests in natural waters and wastewater demonstrate a decrease of the number of cells from 10^8 cells/ml to below 10 cells/ml after 3-4 weeks [10]. Experiments with soil samples indicate a decrease of the number of cells from 10^7/g of soil to a maximum of 100 viable cells within 6-8 weeks, while the naturally occurring bacterial populations in the samples remain constant [11]. Due to their very good characterisation, apathogenicity, and poor survivability in the environment, *E. coli* K12 and its derivatives are approved as part of the biological safety measures defined in Section 6 Clause 4 in conjunction with Annex II Part A of the GenTSV (Genetic Engineering Safety Regulations, Germany).

The strain used in the ARSOlux system is a derivative of the K12 strain with additional defects in the expression of *recA* and *endAI*. The *recA* mutation leads to the inability to homologously recombine DNA, resulting in the stabilisation of transferred recombinant plasmids. The *endAI* mutation considerably reduces endonuclease I activity, facilitating the isolation of DNA from cells. Owing to these two characteristics, DH5α is preferably used for producing recombinant *E. coli* strains [12].

Donor organisms

The gene *arsR* including its promoter was transferred to the DH5α strain. The gene stems from the *E. coli*-specific plasmid R773 [13] and encodes for a transcriptional regulator with binding sites for arsenic compounds. It is a repressor protein bound to its promoter in the absence of arsenic compounds which represses the expression of *downstream* genes. After having been taken up by the bacterial cell arsenic compounds form a complex with ArsR, thereby allosterically preventing the association of ArsR with the promoter. Thus, the transcription of downstream genes is induced. Naturally, these genes are arsenic-resistance determinants. The inserted *arsR* gene with the pertinent promoter has no hazard potential.

Furthermore, reporter genes were inserted into the recipient organisms using a vector. These are the *luxCDABE* genes required for the expression of luciferase from *Photorhabdus luminescens*, an organism of the risk group 1 pursuant to Section 5 Clause 1 in conjunction with Annex I No. 1 of the GenTSV. *P. luminescens* is a representative of *Enterobacteriaceae* and is found in the intestines of entomopathogenic nematodes. The inserted genes have no hazard potential.

Vector

The ARSOlux system employs the pSB403-arsR plasmid in the test bacteria.

The vector background of this plasmid is the pRK415 plasmid [14], which is in turn derived from the RK2 plasmid from *Klebsiella aerogenes*. It contains an *oriV,* which enables a *broad host range* replication in Gram-negative bacteria, an *oriT* to start the plasmid transfer during a triparental conjugation and a tetracycline-resistance gene cartridge. The *luxCDABE* genes were inserted into pRK415 and the resulting plasmid was named pSB403 [15]. The expression of *luxCDABE* genes leads to emission of light.

This vector does not comply with the requirements defined in Section 6 Clause 5 of the GenTSV as being part of the biological safety measures, since no limited host specificity is present. Furthermore, the plasmid can be mobilised if the *tra* and *mob* genes required for

conjugation are provided by what is referred to as a helper strain (triparental conjugation; see below).

DNA insert

A 1 kb *Eco*RI fragment containing the *arsR* gene with the corresponding promoter and two *arsR* binding sites was inserted into the pSB403 vector *upstream* the *luxCDABE* genes [16]. The plasmid was named pSB403-arsR.

Genetically modified organism

E. coli DH5α-2697 is the K12 strain-derived organism into which the recombinant plasmid pSB403-arsR was transferred [17]. When these cells come in contact with arsenic compounds, the compounds enter the cells and bind to the ArsR protein. As a result, the repressor loses its ability to bind to DNA, initiating the expression of the luciferase genes. The intensity of the light emitted is proportional to the concentration of arsenic compounds and can be determined using a luminometer. The recombinant organism constitutes no biological safety measure as defined in Section 6 in conjunction with Annex II Part A of the GenTSV.

Intended use:

The ARSOlux biosensor system is intended to be used as a well-established test system to determine the concentration of arsenic compounds in waters/drinking water plants. The planned studies in Mongolia are supposed to further characterise the effects of water condition (pH value, oxygen content, iron content), ambient temperature and air humidity on measurements. The recombinant bacteria are kept freeze-dried in sealed (septum stopper, aluminium lid) crimp-neck vials (10^{10} - 10^{12} cells). The bacteria are freeze-dried in the presence of a complex organic culture medium (Luria broth, LB), trehalose and the hygroscopic polyvinylpyrrolidone. These vials are stored and transported in Styrofoam boxes containing 100 vials each. Measurements outside a genetic engineering facility are conducted in a measuring vehicle at the sampling location by injecting 1 ml of the water sample to be inspected into the vial using a cannula and subsequently incubating the vial for two hours at a temperature of 20-35°C. To determine the light emission by means of a luminometer (Wallac Victor), the vials are taken out of the box and placed in the luminometer. After conducting the measurement, the vials are put back into the box. The viable, recombinant bacteria in the vials are inactivated by injecting 1 ml of 6% hydrogen peroxide solution. All syringes and cannulas used are collected in break-proof containers, sealed, and labelled. Both the box containing the test vials and the collected syringes and cannulas are subsequently transported to a genetic engineering facility for autoclaving [18].

Assessment

The recombinant bacterial strain used is a derivative of non-pathogenic *E. coli* K12 and complies with the requirements for a recipient organism constituting a biological safety measure as defined in Section 6 Clause 4 of the GenTSV. The nucleic acid segments inserted using the plasmid do not increase the hazard potential of the recipient organism. The recombinant bacteria are allocated to the risk group 1 pursuant to Section 5 Clause 1 in conjunction with Annex I No. 2 of the GenTSV.

Furthermore, the recombinant bacteria are stored in sealed crimp-neck vials, which constitute a closed system. When following the operation manual, it is not assumed that recombinant bacteria would enter the environment.

It should be noted that the pSB403-based plasmid used is a vector that is not only capable of replicating in *E. coli*. The origin of replication contained therein would also enable plasmid

amplification in other Gram-negative bacteria found in soil and water. A transfer of the plasmid to naturally occurring bacteria, however, would require several prerequisites. The crimp-neck vials would have to break due to improper handling and the viable bacterial culture contained therein would have to get in contact with soil or water in the environment. The contaminated soil or water would have to contain bacteria in the immediate vicinity which could serve as helper strains for triparental conjugation. The helper bacteria would have to be capable of complementing the missing mobilisation genes (*mob*, *tra*) in the plasmid used. However, the *mob* genes are specific for plasmid families, since the strand break of the plasmid DNA by means of a nuclease in the *oriT* region, which is required for a transfer, is sequence-specific. In fact, studies on plasmid transfer after mobilisation with comparable plasmid systems suggest that this can only take place under appropriate laboratory conditions. Such transfer was not demonstrated in water samples [19]. The applicant himself has examined the survivability of the used bacteria in unsterile soil. For this purpose, the freeze-dried bacteria in a crimp-neck vial were rehydrated with 1 ml of distilled water, as is done during the measurement process, and incubated for two hours at room temperature before adding this suspension to 15 g of non-sterile soil sample. The samples were incubated at 30°C for a period of 28 days and tested at selected points in time using PCR for the presence of the *luxA* gene contained in the plasmid. In these studies, the *luxA* gene was amplifiable with specific primers only until the 7^{th} day of incubation. These results are comparable to the studies investigating the gene transfer of recombinant *E. coli* K12 strains carrying a pBR-based plasmid [11]. According to the current state of knowledge, the probability of mobilising the plasmid used here is very low, provided that the ARSOlux system is handled properly.

In summary, the use of the ARSOlux system is not considered to present a potential hazard to humans, animals and the environment.

References

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