Notification 6786-01-0071

Summary of the risk assessment of genetically modified sinorhizobia (*Sinorhizobium meliloti)* (derivative L1 and L33 of the strain *S. meliloti* Rm2011) carried out by the German Competent Authority within the framework of a proposed deliberate release, Berlin, 20 August 1997

Explanatory note to this document:

The following text reflects the summary of the risk assessment of (a) genetically modified organism(s) to be used for experimental field trials (deliberate releases) in Germany. The text forms part of the official authorisation regarding applications for the permit of deliberate releases (field trials) of genetically modified organisms in Germany under the legal framework of Directive 2001/18/EC and the German Gene Technology Act (Gentechnikgesetz, GenTG). The authorisation is issued by the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, BVL [*Federal Office of Consumer Protection and Food Safety*], as the German Competent Authority. It comprises the chapters

I. Consent [to the application]
II. Provisions [to be respected in execution of the field trials]
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III.1.1. Requirements for approval according to section 16 (1) Nr. 1 GenTG
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III.1.4. Formal requirements according to section 16 (4, 5) GenTG
III.2 Appraisal of and reply to objections
IV. Costs
V. Legal instruction

Only the original German document is legally binding. The following passage is a courtesy translation of the chapter III.1.2. and was prepared for the Biosafety Clearing-House.

III.1.2.1 Evaluation of changes in the genetically modified plants effected by the transferred nucleic acid sequences

a) The luciferase gene from the firefly *Photinus pyralis*

The cDNA of the luciferase gene codes for the 62 kDa enzyme luciferin 4-monooxygenase, which oxidises the luciferin substrate (a heteropolycyclic organic acid) to oxyluciferin under ATP consumption in the presence of oxygen. This reaction causes the emission of light (bioluminescence), which can be demonstrated by appropriate methods (luminometer, blackening of X-ray films etc.). The luciferase gene of *Photinus pyralis* is used as a reporter gene in various organisms (prokaryotes and eukaryotes). With respect to its molecular composition and substrate specificity, firefly luciferase differs fundamentally from the prokaryotic bioluminescence system from *Vibrio fisheri*.

Published results from previous scientific investigations lead to the expectation that genes related to the firefly gene and conversion of the firefly luciferin do not occur in bacteria. Although it cannot be ruled out that the luciferase could also convert other substances chemically related to the firefly luciferin, a potential activity of the enzyme in *S. meliloti* is not expected to pose a threat to the environment.

b) The *npt*II promoter from the transposon Tn*5* of *E. coli*

The *npt*II promoter was inserted in front of the cDNA sequence to achieve constitutive expression of the *luc* gene in *S. meliloti*. The *nptII* promoter is a bacterial gene regulatory element which occurs in gram-negative bacteria as a component of the *nptII* gene and does not contain any DNA sequence of the *nptII* structural gene.

c) The site of integration of the *luc* gene cassette in *S. meliloti*

The *luc* gene cassette (*npt*II promoter and *luc*-cDNA) was integrated into the chromosome of *S. meliloti* RM2011 by *in vivo* recombination. The method used, by which a double recombination took place, ensures that only the *luc* gene cassette and no vector sequences are integrated into the chromosome of the genetically modified bacteria. DNA hybridisation methods were used to examine the relevant chromosomal regions and confirm correct insertion of the *luc* gene cassette and the absence of vector sequences. The *recA* locus was selected as the site of integration. The *recA* gene codes for a protein that plays a central role in natural recombination processes, DNA repair processes and the control of gene expression following DNA damage. In one of the strains proposed for release, the *recA* gene is inactivated through the integration of the *luc* gene cassette (strain L1); in the other strain, integration into the chromosome took place downstream of the coding region of the *recA* gene (strain L33). Except for the trait conferred by the *luc* gene cassette, the recombination-intact strain L33 does not differ from the original strain. The insertion is not expected to lead to any mutations because the cassette was integrated downstream of the reading frame of the *recA* gene into a non-coding region of the chromosome.

In strain L1, the insertion of the *luc* gene cassette into the coding region led to the inactivation of the *recA* gene. Investigations carried out by the applicant show that *recA* mutants of *S. meliloti* have a prolonged generation time and, in microcosm experiments with non-sterile soil, the L1 strain has a growth disadvantage. Since the *recA* protein plays a central role in DNA repair processes, a prolonged generation time in *recA* mutants is plausible. The planned experiments seek to clarify whether the *recA* mutation leads to a reduced competitive capacity in the environment of the L1 strain compared to the natural microflora. In a deliberate release experiment with the strains L1 and L33, which has been

running since 1994 in Braunschweig, a significantly lower viability of the strain L1 was not detected. This can probably be attributed to the small endogenous *S. meliloti* population at this site. Preliminary investigations at the research site in Straß have shown that there is a much larger endogenous *S. meliloti* population there.

Because the *luc* gene cassette was integrated into the chromosome of both genetically modified bacterial strains, it is expected that the genetic modification will be constantly passed on to progeny and will not have any further effects other than those already mentioned on the characteristics of the GMO. In particular, the genetically modified *S. meliloti* strains are not expected to show altered symbiotic behaviour and be able to interact with plants other than their natural symbiotic partners, because the genes for recognition of symbiotic partners (the so-called nodulation genes) and those genes which are essential for nitrogen fixation (the *nif* and *fix* genes) have remained unaffected.

III.1.2.2. Assessment of the possibility that the genetically modified sinorhizobia might spread beyond the experimental area

Bacteria of the genus *Sinorhizobium* are gram-negative, aerobic soil bacteria which can form a symbiosis with legumes and, in that symbiosis, incorporate molecular nitrogen into organic compounds. The presence of host plants is crucial for the propagation of *Sinorhizobium* because effective propagation of these bacteria takes place in soil in the rhizosphere of the symbiotic plants. (The rhizosphere is defined as the root surface and that portion of the soil which is directly influenced by the root system.) Previous investigations in the field with genetically marked *Sinorhizobium* bacteria showed that the lateral and vertical spread of the bacteria is largely linked to the root development of the host plants. The close socialisation between *Sinorhizobium* and the roots of its symbiotic partner means that its transfer by wind or rainwater alone do not represent effective dispersal pathways for these bacteria.

The species intended for the deliberate release experiment, *S. meliloti,* has a narrow host range and only enters into symbioses with legumes of the genera *Medicago* (lucerne), *Melilotus* (melilot, sweet clover) and *Trigonella* (fenugreek). Since no lucerne or other host plants of *S. meliloti* are cultivated in the neighbourhood (up to 100 m) of the test ground, the preconditions for propagation of the GMO outside the test ground are extremely limited (see supplementary provision II.8).

Any naturally occurring host plants of *S. meliloti* growing in the vicinity of the test ground are to be included in the investigations to check for a potential spread of the GMO (see, on this matter, supplementary provision II.8). The planned monitoring measures are intended to ensure early identification of any unexpected spread of the GMO. The removal, if necessary, of symbiotic partners from the vicinity of the testing ground is a measure to ensure that the trial is confined.

Although a carryover of the GMO into the groundwater through leachate is possible, it is important to note that it is highly likely that sinorhizobia, as symbiotic soil bacteria, would not be able to persist in an aqueous environment but would be displaced by the normal microflora in water. A passive horizontal spread of genetically modified bacteria beyond the release plots by rainwater cannot be ruled out either because the testing ground exhibits a slight gradient. The above-described monitoring programme ensures that any potential spread, also beyond the testing ground, can be tracked. Basically, it is important to note that any spread of sinorhizobia takes place essentially on the basis of its interaction with the host plant. This was verified in the deliberate release trial with the genetically modified

bacteria described here in Braunschweig. One year after application/release, in places outside the plots where no lucerne grew, only GMOs with a live titre close to the detection limit were found.
The deliberate release plots are located on the grounds of the *Staatliche Versuchsgüterverwaltung Freising* (research facility of the federal state of Bavaria) which are surrounded by a wild animal protection fence. As a result, wild animals are kept away and accidental entry by unauthorised persons is not expected. This measure is considered sufficient to minimise the unintentional transfer of GMOs by persons or wild animals.
Objects (footwear, tools, etc.) that may come into contact with GMOs within the scope of the experimental work (e.g. application of the GMOs, taking of samples) are to be cleaned after the end of the work on the testing ground to minimise the carryover of GMOs (see supplementary provision II.5).

III.1.2.3. Evaluation of the ability of the genetically modified sinorhizobia to establish on the deliberate release plots

The rhizosphere of the host plant, with which a symbiosis can be formed, is crucially important for the effective propagation of the sinorhizobia. The generation time of sinorhizobia in the soil outside the rhizosphere amounts to more than 200 hours, whereas the generation time in the rhizosphere is estimated to be 9 to 12 hours. Permanent states, such as spores, are not formed by sinorhizobia. Over several decades, sinorhizobia were applied worldwide on a large scale to improve the growth of legumes; sinorhizobia that were introduced into the soil for this purpose were thereby found to decrease their titre in the absence of host plants.
In the present deliberate release experiment, the genetically modified *Sinorhizobium* strains are to be applied in plots in 1997. The quantity of GMOs to be released is calculated such that up to a depth of 25 cm a bacterial titre of 106 bacteria per gram of soil is reached. After the bacteria have been applied, their symbiotic partner lucerne (*Medicago sativa*) will be planted on the 49 plots. In 21 of those plots, the lucerne will be removed in the second year of the trial, the soil will be turned and grain will be sown. The lucerne will be harvested up to three times per year, the grain once per year, whereby the harvested plants are to be composted on the test ground. The applicant does not plan to remove any potentially remaining genetically modified sinorhizobia from the release plots after completion of the trial.
On the basis of experience gained from releases of sinorhizobia, it is assumed that the genetically modified bacteria can persist in the area of the rhizosphere of the lucerne roots. In the soil areas of the plots which do not belong to the rhizosphere of the host plants, no effective propagation of the GMO is expected. Consequently, the initial GMO titre is expected to decrease in these soil areas.
Because *S. meliloti* depends on the presence of its symbiotic partner lucerne for efficient propagation in soil, the measures required by supplementary provision II.9 (destruction of the capacity for growth of the host plants, monitoring of the GMO titre) after completion of the test periods are suitable for precautionary restriction of the trial. The GMO titre is expected to decrease on the trial plots after the growth capacity of the host plants has been destroyed. Also, to enable detection of any unexpected increase in the population of the GMO, monitoring of the GMO titre after destruction of the growth capacity of the host plants has been prescribed. If this monitoring activity shows any evidence of an unexpected increase in the population of the GMO, measures to eliminate the GMO would be required.

In the case of a decrease in the GMO titre in the plots, measures to eliminate the GMO are dispensable because according to the risk assessment of the genetically modified organisms, a threat to the legal interests specified under § 1 No.1 of the German Gene Technology Act (GenTG) is not expected.

III.1.2.4. Assessment of the possibility of transfer of the inserted foreign gene from the genetically modified sinorhizobia to other microorganisms by horizontal gene transfer

The inserted DNA sequence was stably integrated into the chromosome of the recipient organism by *in vivo* recombination. The integration of the *luc* gene cassette into the chromosome of the GMO makes a transfer of the sequence by conjugative processes unlikely. Chromosomal genes can, in principle, be transferred by means of bacteriophage-mediated transduction. Solid scientific findings concerning the frequency and significance of this form of gene transfer in the natural environment are so far not available for sinorhizobia. The *luc* gene cassette could be transferred to other organisms through the release of chromosomal DNA from dead GMOs and the uptake of the pure nucleic acids by other soil bacteria. Such transformation processes are conceivable in soil bacteria which have a natural competence to uptake DNA. These also include *S. meliloti* and other soil bacteria. For the integration of DNA taken up by homologous recombination into the DNA of a recipient organism, homology of the DNA sequences flanking the cassette must also be present. It is not known whether transformation, i.e. the uptake of free DNA and potential establishment of this DNA, makes a significant contribution to gene transfer under natural conditions.

The chances of a potential transfer of the *luc* gene cassette to other organisms are extremely low, but nevertheless conceivable. But this possibility exists for all DNA irrespective of its origin and not in particular for the *luc* gene cassette. There is no reason to assume that the *luc* gene cassette can confer a selective advantage on *S. meliloti* or other soil microorganisms. A spread of the *luc* gene cassette beyond the proposed deliberate release through macroorganisms is not anticipated. There are no risk-inducing traits associated with the *luc* gene cassette. Even if the *luc* gene cassette were to be transferred to other soil bacteria, there is nothing to indicate that this could represent a hazard.