PART 1 (COUNCIL DECISION 2002/813/EC)

SUMMARY NOTIFICATION INFORMATION FORMAT FOR THE RELEASE OF GENETICALLY MODIFIED ORGANISMS OTHER THAN HIGHER PLANTS IN ACCORDANCE WITH ARTICLE 11 OF DIRECTIVE 2001/18/EC

In order to tick one or several possibilities, please use crosses (meaning x or X) into the space provided as (.)

A. General information

1. Details of notification

(a)	Member State of notification	Sweden
(b)	Notification number	B/SE/05/KEMI-723-573
(c)	Date of acknowledgement of notification:	05/04/2005

(d) Title of the project:

Risk assessment of non-resident microorganisms used for biocontrol of fungal pathogens.

Proposed period of release From 08/2005 until Summer 2006

2. Notifier

Name of institution or company:

Dept of Microbiology
Swedish University of Agricultural Sciences - SLU
Box 7025
750 07 Uppsala, Sweden

3. GMO characterisation

(a) Indicate whether the GMO is a:

viroid	(.)
RNA virus	(.)
DNA virus	(.)
bacterium	(X)
fungus	(.)
animal	
- mammals	(.)
- insect	(.)
- fish	(.)
- other animal	(.)
specify phylum, class	…

(b) **Identity of the GMO (genus and species)**

Pseudomonas fluorescens

(c) **Genetic stability – according to Annex IIIa, II, A(10)**

No evidence has been obtained to suggest that SBW25 is any more or less genetically stable than other fluorescent pseudomonads. By FAME-MIS analysis populations of SBW25, inoculated onto glasshouse plants, demonstrate no distinguishable variation. Data obtained by repeated sub-culture of the recombinant in non-selective broth for 100 generations confirmed the genetic stability of the recipient.

SBW25:*tgl* has been chromosomally marked using a disarmed transposon vector. Disarmed transposons are very suitable for marking gram-negative bacteria.

4. Is the same GMO release planned elsewhere in the Community (in conformity with Article 6(1)), by the same notifier?

Yes	(.)	No	(X)
If yes, insert the country code(s)		…	

5. Has the same GMO been notified for release elsewhere in the Community by the same notifier?

	Yes (.)	No	(X)
If yes:			
- Member State of notification		…	
- Notification number		B/../../…	

Please use the following country codes:

Austria AT; Belgium BE; Germany DE; Denmark DK; Spain ES; Finland FI; France FR; United Kingdom GB; Greece GR; Ireland IE; Iceland IS; Italy IT; Luxembourg LU; Netherlands NL; Norway NO; Portugal PT; Sweden SE

6. Has the same GMO been notified for release or placing on the market outside the Community by the same or other notifier?

	Yes (.)	No	(X)
If yes:			
- Member State of notification		…	
- Notification number		B/../../…	

7. Summary of the potential environmental impact of the release of the GMOs.

The organism *P. fluorescens* is a natural member of the rhizosphere bacterial communities on wheat and other plants. The strain SBW25 was originally isolated from sugar beet in the UK. SBW25:*tgl* is expected to behave identically to the wild type SBW25 on field grown plants. The marker genes give no selective advantages to the GMM. SBW25 is sensitive to cold and will probably not survive well in the Swedish winter. The effects on non-target microorganisms in this project will most probably be transient. One aim of this field trial is to investigate the possible impacts on non-target fungi and bacteria. None of these possible effects is thought to be irreversible. No difference between the SBW25:*tgl* and the wild-type organism is expected.

The bacterium will be used as a plant growth-promoting strain on wheat by seed treatment. The *telAB/kilA/gfp/luxAB* genes have been inserted into the chromosome of *P. fluorescens* SBW25:*tgl* for monitoring purposes. This combination of marker genes gives an ideal combination for observing released bacteria in field trials. SBW25 is an efficient rhizosphere

colonizer, which is a necessary trait for a biocontrol agent. *Pseudomonas* spp. are not known to produce long term survival structures. The bacterium SBW25 has been extensively studied in laboratory, greenhouse and field trials. None of the inserted genes produce substances that are harmful to humans, animals or plants. The wild-type bacterium is not pathogenic to humans, animals or plants.
A few bacterial cells may spread by wind and water from the release site. It is however unlikely that this will result in established, active populations of SBW25:*tgl*.

B. Information relating to the recipient or parental organism from which the GMO is derived

1. Recipient or parental organism characterization:

(a) Indicate whether the recipient or parental organism is a:

(select one only)

	viroid	(.)
	RNA virus	(.)
	DNA virus	(.)
	bacterium	(X)
	fungus	(.)
	animal	
-	mammals	(.)
-	insect	(.)
-	fish	(.)
-	other animal	(.)
	(specify phylum, class)	…

other, specify …

2. Name

(i)	order and/or higher taxon (for animals)	…
(ii)	genus	*Pseudomonas*
(iii)	species	*fluorescens*
(iv)	subspecies	…
(v)	strain	SBW25
(vi)	pathovar (biotype, ecotype, race, etc.)	…
(vii)	common name	…

3. Geographical distribution of the organism

(a) Indigenous to, or otherwise established in, the country where the notification is made:

Yes (.) No (.)
Not known (X)

(b) Indigenous to, or otherwise established in, other EC countries:

(i) Yes (X)

If yes, indicate the type of ecosystem in which it is found:

Agicultural field in United Kingdom.

(ii) No (.)

(iii) Not known (.)

(c) Is it frequently used in the country where the notification is made?

Yes (X) No (.)

(d) Is it frequently kept in the country where the notification is made?

Yes (X) No (.)

4. Natural habitat of the organism

(a) If the organism is a microorganism

water	(.)
soil, free-living	(.)
soil in association with plant-root systems	(X)
in association with plant leaf/stem systems	(.)
other, specify …	

(b) If the organism is an animal: natural habitat or usual agroecosystem:

N/A

5. (a) Detection techniques

Pseudomonas selective agar: with CFC (cephaloridine, fucidin, and cetrimide) supplemented can be used to isolate nonmodified SBW25 and other fluorescent pseudomonads.

(b)

Identification techniques:

PCR amplification: by using 16S specific primers for SBW25.

6. Is the recipient organism classified under existing Community rules relating to the protection of human health and/or the environment?

Yes (X) No (.)

If yes, specify: *P. fluorescens* belong to risk group 1 (the lowest one) according to European Community classification Directive 90/679/EEC on the protection of workers from risks to exposure to biological agents at work. Group 1 = A biological agent that is most unlikely to cause human disease.

7. Is the recipient organism significantly pathogenic or harmful in any other way (including its extracellular products), either living or dead?

Yes (X) No (.) Not known (.)

The bacterium SBW25:*tgl* is not a known plant pathogen or recognized vertebrate pathogen.

If yes:

(a) to which of the following organisms:

humans (.)
animals (.)
plants (.)
other (X) fungi

(b) give the relevant information specified under Annex III A, point II. (A)(11)(d) of Directive 2001/18/EC

The bacterium SBW25:*tgl* is not a known plant pathogen or recognized vertebrate pathogen. It is not able to multiply in warm-blooded animals. After seed inoculation of a variety of plants, SBW25 was detected on sampled leaves and roots. It will be used to inhibit plant-pathogenic fungi.

8. Information concerning reproduction

(a) Generation time in natural ecosystems:

The generation times in natural ecosystems are difficult to assess and depends on nutrient supplies, pH, inhibiting substances, water potential, competition and temperature. Mean generation time of SBW25:*tgl* at 25 ^{o}C in L-broth determined at 1 h. *In situ* estimates of growth rate have been extrapolated from glasshouse grown inoculated plants. Maximal rates for SBW25:*tgl* mean generation time on the plant surface are assumed to be in the order of 2-5h.

(b) Generation time in the ecosystem where the release will take place:

The generation times in natural ecosystems are difficult to assess and depends on nutrient supplies, pH, inhibiting substances, water potential, competition and temperature. Mean generation time of SBW25:*tgl* at 25 ^{o}C in L-broth determined at 1 h. *In situ* estimates of growth rate have been extrapolated from glasshouse grown inoculated plants. Maximal rates for SBW25:*tgl* mean generation time on the plant surface are assumed to be in the order of 2-5h.

(c) Way of reproduction:

Sexual (..) Asexual (X)

(d) Factors affecting reproduction:

Temperature, water availability, pH, nutrients, competition, inhibiting substances

9. Survivability

(a) ability to form structures enhancing survival or dormancy:

(i)	endospores	(.)
(ii)	cysts	(.)
(iii)	sclerotia	(.)
(iv)	asexual spores (fungi)	(.)
(v)	sexual spores (funghi)	(.)
(vi)	eggs	(.)
(vii)	pupae	(.)
(viii)	larvae	(.)
(ix)	other, specify …	

(b) Relevant factors affecting survivability:

Pseudomonas spp. are not known to produce long term survival structures, but they are polymorphic and thus are able to tolerate adverse environmental conditions. The recipient organism is a natural member of the rhizosphere bacterial communities on wheat and other plants in the UK. The strain SBW25 is sensitive to cold and might not survive the Swedish winter. SBW25:*tgl* is expected to behave identically to the wild type SBW25 on field grown plants. The marker genes give no selective advantages to the GMM.

10. (a) Ways of dissemination

A few bacterial cells may spread by wind and water from the release site. Rain splashes could carry the organism short distances (dm) from leaves and maybe from the soil. SBW25:*tgl* could disperse through surface contact with colonized plants. Studies on sugar beet demonstrate that flying insects can be contamined with GMMs after landing on colonized leaves. Thus any organism (birds, mammals etc) could act as a vector of the GMM (or any other microorganism).

It is however unlikely that these ways will result in large, active populations of SBW25:*tgl*.

(b) Factors affecting dissemination

1. Temperature: Optimum growth conditions 25-30 ˚C. No growth occurs below 4 ˚C or above 37 ˚C and the organism will die within four days. At temperatures below freezing the bacterium is killed.
2. Competitions, predation and parasitism: Experiments has shown that the recombinant or wild type cannot survive on decaying wheat roots at 100 % RH at 25 ˚C in competition with indigenous microorganisms.
3. pH: Neutral to slightly alkali conditions is favorable.
4. Nutrition: SBW25:tgl takes up nutrients from plant exudates and other easily degradable carbon sources.
5. Humidity: Multiplication can only take place when enough moisture is available (100 % RH).

11. Previous genetic modifications of the recipient or parental organism already notified for release in the country where the notification is made (give notification numbers)

No.

C. Information relating to the genetic modification

1. Type of the genetic modification

(i)	insertion of genetic material	(X)
(ii)	deletion of genetic material	(.)
(iii)	base substitution	(.)
(iv)	cell fusion	(.)
(v)	others, specify …	

2. Intended outcome of the genetic modification

The bacterium was chromosomally tagged with genes *kilA, telAB* conferring resistance to potassium tellurite, the constitutive promoter *PpsbA*, the *gfp* gene producing green fluorescent protein and *luxAB* genes encoding bioluminescence production. All these genes were inserted in order to monitor and trace the bacterium in the wheat plant-soil environment.

3. (a) Has a vector been used in the process of modification?

Yes (X) No (.)

If no, go straight to question 5.

(b) If yes, is the vector wholly or partially present in the modified organism?

Yes (X) No (.)

If no, go straight to question 5.

4. If the answer to 3(b) is yes, supply the following information

(a) Type of vector

plasmid	(.)
bacteriophage	(.)
virus	(.)
cosmid	(.)
transposable element	(X)
other, specify	…

(b) Identity of the vector

The vector is based upon the miniTn5-transposon containing resistance to potassium tellurite (non-antibiotic selection) with the *gfp/luxAB* genes added at the unique *Not*I site.

(c) Host range of the vector

Gram-negative bacteria.

(d) Presence in the vector of sequences giving a selectable or identifiable phenotype

Yes (X) No (.)

antibiotic resistance	(.)
other, specify	resistance to potassium tellurite (K_2TeO_3)

Indication of which antibiotic resistance gene is inserted
N/A

(e) Constituent fragments of the vector

kilA, telAB: The tellurite resistance is encoded by the cryptic *tel*AB and *kil*A genes of plasmid RK2 from *Klebsiella aerogenes*.
PpsbA/gfp/luxAB: The *PpsbA/gfp/luxAB* genes were excised from plasmid pUTgfplux as a *Not*I fragment and ligated into the unique *NotI*-site of pUTtel.

(f) Method for introducing the vector into the recipient organism

(i)	transformation	(.)
(ii)	electroporation	(X)
(iii)	macroinjection	(.)
(iv)	microinjection	(.)
(v)	infection	(.)
(vi)	other, specify …	

5. If the answer to question B.3(a) and (b) is no, what was the method used in the process of modification?

(i)	transformation	(.)
(ii)	microinjection	(.)
(iii)	microencapsulation	(.)
(iv)	macroinjection	(.)
(v)	other, specify	…

6. Composition of the insert

(a) Composition of the insert

kilA, telAB: The tellurite resistance is encoded by the cryptic *tel*AB and *kil*A genes of plasmid RK2 from *Klebsiella aerogenes*.
PpsbA/gfp/luxAB: The *PpsbA/gfp/luxAB* genes were excised from plasmid pUTgfplux as a *Not*I fragment and ligated into the unique *NotI*-site of pUTtel.

(b) Source of each constituent part of the insert

kilA, telAB: *Klebsiella aerogenes* (bacterium)
PpsbA: *Amaranthus hybridus* (pigweed)
gfp: *Aequorea victoria* (small jellyfish)
luxAB: *Vibrio harveyi* (bacterium)

(c) Intended function of each constituent part of the insert in the GMO

kilA, telAB: these genes confer resistance to the chemical compound potassium tellurite (K_2TeO_3). The use of these genes as marker genes was developed to suit strains that were aimed to be released in field trials, where it is not desirable to use antibiotic resistance genes as marker tools for tracking the bacteria. Makes selective plating on potassium tellurite possible.
PpsbA: a constitutive promoter for the expression of *gfp* and *luxAB*.

gfp: encoding green fluorescent protein (GFP). GFP emits green light at 508 nm upon ultraviolet light illumination at 396 nm, no additional substrates needed. GFP is a measurement of the total number of cells since it is expressed regardless of metabolic activity in the cells.
luxAB: encoding bacterial luciferase. The phenotype, bioluminescence, is dependent on the energy reserves of the cells, $FMNH_2$. Therefore, the *luxAB* genes can be used to determine the number of metabolically active cells. Bioluminescence requires *n*-decanal as a substrate.

(d) Location of the insert in the host organism

-	on a free plasmid	(.)
-	integrated in the chromosome	(X)
-	other, specify	…

(e) Does the insert contain parts whose product or function are not known?

Yes (.) No (X)

If yes, specify …

D) a. Information on the organism from which the *gfp* gene is derived

***gfp* gene**

1. Indicate whether it is a:

viroid	(.)
RNA virus	(.)
DNA virus	(.)
bacterium	(.)
fungus	(.)
animal	
- mammals	(.)
- insect	(.)
- fish	(.)
- other animal	(X)
(specify phylum, class)	…
other, specify	jellyfish

2. Complete name

(i)	order and/or higher taxon (for animals)		…
(ii)	family name for plants		…
(iii)	genus	*Aequorea*	
(iv)	species	*victoria*	
(v)	subspecies		…
(vi)	strain		…
(vii)	cultivar/breeding line		…
(viii)	pathovar		…

(ix) common name jellyfish

3. Is the organism significantly pathogenic or harmful in any other way (including its extracellular products), either living or dead?

Yes (.) No (X) Not known (.)

If yes, specify the following:

(a) to which of the following organisms:

humans (.)
animals (.)
plants (.)
other ..

(b) are the donated sequences involved in any way to the pathogenic or harmful properties of the organism

Yes (.) No (X) Not known (.)

If yes, give the relevant information under Annex III A, point II(A)(11)(d):
…

4. Is the donor organism classified under existing Community rules relating to the protection of human health and the environment, such as Directive 90/679/EEC on the protection of workers from risks to exposure to biological agents at work?

Yes (.) No (X)

If yes, specify …

5. Do the donor and recipient organism exchange genetic material naturally?

Yes (.) No (X) Not known (.)

D) b. Information on the organism from which the *luxAB* genes are derived

***luxAB* genes**

1. Indicate whether it is a:

viroid (.)
RNA virus (.)
DNA virus (.)
bacterium (X)
fungus (.)
animal
- mammals (.)
- insect (.)
- fish (.)
- other animal (.)

(specify phylum, class) …
other, specify …

2. Complete name

(j)	order and/or higher taxon (for animals)	…
(ii)	family name for plants	…
(iii)	genus	*Vibrio*
(iv)	species	*harveyi*
(v)	subspecies	…
(vi)	strain	…
(vii)	cultivar/breeding line	…
(viii)	pathovar	…
(ix)	common name	

3. Is the organism significantly pathogenic or harmful in any other way (including its extracellular products), either living or dead?

Yes (X) No (.) Not known (.)

Vibrio harveyi are straight or curved bacterial rods, gram negative with polar flagellae. Found in aquatic habitats with a wide range of salinity. Pathogenic to vertebrates, opportunistic pathogens to humans.

If yes, specify the following:

(a) to which of the following organisms:

humans (.)
animals (X)
plants (.)
other ..

If yes, give the relevant information under Annex III A, point II(A)(11)(d):

(b) are the donated sequences involved in any way to the pathogenic or harmful properties of the organism

Yes (.) No (X) Not known (.)

4. Is the donor organism classified under existing Community rules relating to the protection of human health and the environment, such as Directive 90/679/EEC on the protection of workers from risks to exposure to biological agents at work?

Yes (X) No (.)

If yes, specify: Group 2: A biological agent that may cause human disease and which might be a hazard to laboratory workers but is unlikely to spread in the community. Laboratory exposure rarely produces infection and effective prophylaxis or treatment is available.

5. Do the donor and recipient organism exchange genetic material naturally?

Yes (.) No (.) Not known (X)

D) c. Information on the organism from which the *kilA, telAB* genes are derived

***kilA, telAB* genes**

1. Indicate whether it is a:

viroid	(.)
RNA virus	(.)
DNA virus	(.)
bacterium	(X)
fungus	(.)
animal	
- mammals	(.)
- insect	(.)
- fish	(.)
- other animal	(.)
(specify phylum, class)	…
other, specify	…

2. Complete name

(k)	order and/or higher taxon (for animals)		…
(ii)	family name for plants		…
(iii)	genus	*Klebsiella*	
(iv)	species	*aerogenes*	
(v)	subspecies		…
(vi)	strain		…
(vii)	cultivar/breeding line		…
(viii)	pathovar		…
(ix)	common name		

3. Is the organism significantly pathogenic or harmful in any other way (including its extracellular products), either living or dead?

Yes (X) No (.) Not known (.)

Klebsiella aerogenes are gram-negative, straight rods that are nonmotile and facultatively anaerobic. They may be found in intestinal contents, clinical specimens, soil, water, grain etc. They are considered as opportunistic pathogens.

If yes, specify the following:

(a) to which of the following organisms:

humans	(.)
animals	(X)
plants	(.)
other	..

(b) are the donated sequences involved in any way to the pathogenic or harmful properties of the organism

Yes (.) No (X) Not known (.)

If yes, give the relevant information under Annex III A, point II(A)(11)(d):

4. Is the donor organism classified under existing Community rules relating to the protection of human health and the environment, such as Directive 90/679/EEC on the protection of workers from risks to exposure to biological agents at work?

Yes (X) No (.)

If yes, specify: Group 2: A biological agent that may cause human disease and which might be a hazard to laboratory workers but is unlikely to spread in the community. Laboratory exposure rarely produces infection and effective prophylaxis or treatment is available.

5. Do the donor and recipient organism exchange genetic material naturally?

Yes (.) No (.) Not known (X)

E. Information relating to the genetically modified organism

1. Genetic traits and phenotypic characteristics of the recipient or parental organism which have been changed as a result of the genetic modification

(a) is the GMO different from the recipient as far as survivability is concerned?

Yes (.) No (X) Not known (.)

Specify …

(b) is the GMO in any way different from the recipient as far as mode and/or rate of reproduction is concerned?

Yes (.) No (X) Unknown (.)

Specify …

(c) is the GMO in any way different from the recipient as far as dissemination is concerned?

Yes (.) No (X) Not known (.)

Specify …

(d) is the GMO in any way different from the recipient as far as pathogenicity is concerned?

Yes (.) No (X) Not known (.)

Specify …

2. Genetic stability of the genetically modified organism

No evidence has been obtained to suggest that SBW25 is any more or less genetically stable than other fluorescent pseudomonads. By FAME-MIS analysis populations of SBW25, inoculated onto glasshouse plants, demonstrate no distinguishable variation. Data obtained by repeated sub-culture of the recombinant in non-selective broth for 100 generations confirmed the genetic stability of the recipient.

3. Is the GMO significantly pathogenic or harmful in any way (including its extracellular products), either living or dead?

Yes (X) No (.) Unknown(.)

The bacterium SBW25:*tgl* is not a known plant pathogen or recognized vertebrate pathogen.

(a) to which of the following organisms?

humans (.)
animals (.)
plants (.)
other (X) fungi

(b) give the relevant information specified under Annex III A, point II(A)(11)(d) and II(C)(2)(i)

The bacterium SBW25:*tgl* is not a known plant pathogen or recognized vertebrate pathogen. It is not able to multiply in warm-blooded animals. After seed inoculation of a variety of plants, SBW25 was detected on sampled leaves and roots. It will be used as inhibitor of plant-pathogenic fungi.

4. Description of identification and detection methods

(a) Techniques used to detect the GMO in the environment

Selective plating (resistance to potassium tellurite): The resistance to potassium tellurite gives a way of quantifying the amount of SBW25:*tgl* cells by plating on agar plates containing potassium tellurite. Tel^R-positive bacterial colonies appears as black colonies due to the conversion of TeO_2^{2-} to metallic tellurium. Resistance to tellurite has been developed as a way of avoiding antibiotic resistance genes in environmental field trials.

Flow cytometry (green fluorescent protein): allows fluorescent cells to be easily detected in complex environmental samples. Flow cytometry has earlier been used to monitor SBW25:gfp/lux in soil samples.

Fluorescence stereomicroscopy (green fluorescent protein): is used to visualize fluorescent bacteria by eye under blue-light illumination as micro-colonies on plant material and on agar plates.

Epifluorescence microscopy (green fluorescent protein: is used to study gfp-tagged cells with a magnification up to 1000 X.

Confocal scanning laser microscopy (green fluorescent protein): is an imaging technique based on fluorescence that provides greater resolution than standard fluorescence microscopy. CSLM scans one focus layer at a time and if these layers are saved, software can be used to create three-dimensional images of the plant tissues thus displaying the *gfp*-tagged bacteria inside or on the surface of plants.

Luminometry (bioluminescence): is used to measure luciferase activity from metabolically active cells. The phenotype of the expressed *luxAB* genes, encoding luciferase, is light production, bioluminescence. Bioluminescence, which requires *n*-decanal as a substrate, is quantified in a luminometer. No bioluminescence is produced without addition of *n*-decanal. Detectors measure the emitted light and light intensity is proportional to the number of cells in the sample and their metabolic activity.

(b) Techniques used to identify the GMO

PCR amplification: of introduced genes or by using 16S specific primers for SBW25.

Fluorescence stereomicroscopy (green fluorescent protein): due to the specificity of the *gfp* gene this method can also be used for identification.

Epifluorescence microscopy (green fluorescent protein: is used to study gfp-tagged cells with a magnification up to 1000 X.

Flow cytometry (green fluorescent protein): due to the specificity of the *gfp* gene this method can also be used for identification.

Confocal scanning laser microscopy (green fluorescent protein): is an imaging technique based on fluorescence that provides greater resolution than standard fluorescence microscopy. CSLM scans one focus layer at a time and if these layers are saved, software can be used to create three-dimensional images of the plant tissues thus displaying the *gfp*-tagged bacteria inside or on the surface of plants.

Luminometry (bioluminescence): due to the specificity of the *luxAB* genes this method can also be used for identification.

F. Information relating to the release

1. Purpose of the release (including any significant potential environmental benefits that may be expected)

The bacterium will be used as a plant growth-promoting strain on wheat. The *telAB/kilA/gfp/luxAB* genes have been inserted into the chromosome of *P. fluorescens* SBW25:*tgl* for monitoring purposes. The bacterium has been genetically modified in order to make possible tracking and monitoring in the soil and plant environments. This combination of marker genes gives an ideal combination for observing released bacteria in field trials. SBW25:*tgl* is an efficient rhizosphere colonizer, which is a necessary trait for a biocontrol agent.

It is proposed to study the environmental effects on indigenous microorganisms of *P. fluorescens* SBW25:*tgl*. The field trial will evaluate the possible risks of introducing a biological control agent into an agricultural field. Potential effects on indigenous bacterial and fungal populations will be evaluated regarding negative ecological effects. The impact of the inoculum on denitrification will be estimated using DGGE and conserved primers for the functional gene *nosZ*. Active microbial cells (both bacteria and fungi) in soil and on plants will be tagged with Bromodeoxyuridine, a thymidine analogue, and the active DNA will be extracted which will correspond to the active microbes in the environment. T-RFLP will be used with general and specific primers for each group, measuring the impact of the inoculum on key functional groups of bacteria and fungi. Selective plating will be done on mercury and

potassium tellurite to assess the risk of plasmid uptake into the SBW25:*tgl* cells from indigenous microorganisms. Mercury resistance is a common plasmid-encoded trait.

2. Is the site of the release different from the natural habitat or from the ecosystem in which the recipient or parental organism is regularly used, kept or found?

Yes (.) No (X)

If yes, specify …

3. Information concerning the release and the surrounding area

(a) Geographical location (administrative region and where appropriate grid reference):

The release will take place in the municipality of Uppsala.

(b) Size of the site (m^2): 94.5 m^2

(i) actual (GMM) release site (m^2): 3 m^2

(ii) wider (bacterial non-GMM) release site (m^2): 6 m^2

(c) Proximity to internationally recognized biotopes or protected areas (including drinking water reservoirs), which could be affected:

A small brook flows about 200 m away from the site.

(d) Flora and fauna including crops, livestock and migratory species which may potentially interact with the GMO

The fauna of the sites is typical of agricultural land. The surrounding habitats include mixed agriculture (meadows, arable crops and woodland). A fence will protect the plots from large herbivores. Birds and insects. A bird net will protect the seeds from birds.

4. Method and amount of release

(a) Quantities of GMOs to be released:

Approximately 0,25 kg wheat seeds are needed for 12 m^2. That will require 16,7 ml cell suspension of 10^9 cells/ml PBS that corresponds to 1,67 x 10^{10} cells in total.

(b) Duration of the operation:

August 2005 – Summer 2006

(c) Methods and procedures to avoid and/or minimize the spread of the GMOs beyond the site of the release

Immediately after sowing the plots will be covered with a plastic cover to protect the seeds from birds and to retain the moisture after the sowing by hand. The plastic will be removed as soon as germinating has begun. The plastic will be incinerated. A fence will protect the plots from large herbivores and from intrusion by unauthorized individuals. A bird net will protect the seeds from birds.

Boots etc will be dipped for decontamination in a 1% solution of hypochlorite after leaving the release site as part of the normal experimental practice, to prevent unnatural

spread to the surrounding environment. Excess sample material dilution series, agar plates, wash water, enrichment broth etc. will be autoclaved.

5. Short description of average environmental conditions (weather, temperature, etc.)

The site is situated in the mid-east of Sweden, near Uppsala. Climatic conditions in these regions can be described as a temperate climate. Yearly rainfall is typically around 1000 mm ,and average temperatures are around – 6 ^{O}C in winter and 16 ^{O}C in summer.

6. Relevant data regarding previous releases carried out with the same GMO, if any, specially related to the potential environmental and human health impacts from the release.

…

G. Interactions of the GMO with the environment and potential impact on the environment, if significantly different from the recipient or parent organism

The GMM is not significantly different from the wild-type.

1. Name of target organism (if applicable)

(i) order and/or higher taxon (for animals)…
(ii) family name for plants …
(iii) genus …
(iv) species …
(v) subspecies …
(vi) strain …
(vii) cultivar/breeding line …
(viii) pathovar …
(ix) common name …

2. Anticipated mechanism and result of interaction between the released GMOs and the target organism (if applicable)

SBW25:*tgl* has plant growth-promoting traits presumably by biocontrol of fungal infections. The mechanism is thought to be complex with a combination of competition for niches on the root surface, plant growth stimulation and fungal antagonism.

3. Any other potentially significant interactions with other organisms in the environment

SBW25:*tgl* will interact with other microorganisms in the environment. One of the aims of this study is to evaluate the impact of the inoculum on the ecologically important bacteria and fungi. The possible effects are most likely to be transient but it still could affect the soil environment and thus the growing plants.

4. Is post-release selection such as increased competitiveness, increased invasiveness for the GMO likely to occur?

Yes (.) No (X) Not known (.)

Give details

…

5. Types of ecosystems to which the GMO could be disseminated from the site of release and in which it could become established

Pseudomonas fluorescens has a worldwide distribution in soil, water and plant environments. The strain *P. fluorescens* SBW25 is a colonizer of wheat and some other plant species. It colonizes the rhizosphere, roots and phytosphere but is not able to survive in soil for longer periods.

A few bacterial cells may spread by wind and water from the release site. Rain splashes could carry the organism short distances (dm) from leaves and maybe from the soil. SBW25:*tgl* could disperse through surface contact with colonized plants. Studies on sugar beet demonstrate that flying insects can be contamined with GMMs after landing on colonized leaves. Thus any organism (birds, mammals etc) could act as a vector of the GMM (or any other microorganism).

It is however unlikely that these ways will result in established, active populations of SBW25:*tgl*.

6. Complete name of non-target organisms which (taking into account the nature of the receiving environment) may be unintentionally significantly harmed by the release of the GMO

It is proposed to study the environmental effects on indigenous microorganisms of the non pathogenic, plasmid free bacterium called *Pseudomonas fluorescens* SBW25:*tgl*.

SBW25:*tgl* will interact with other microorganisms in the environment. One of the aims of this study is to evaluate the impact of the inoculum on the ecologically important bacteria and fungi. The possible effects are most likely to be transient but it still could affect the soil environment and thus the growing plants.

(i) order and/or higher taxon (for animals)…
(ii) family name for plants …
(iii) genus …
(iv) species …
(v) subspecies …
(vi) strain …
(vii) cultivar/breeding line …
(viii) pathovar …
(ix) common name …

7. Likelihood of genetic exchange in vivo

(a) from the GMO to other organisms in the release ecosystem:

No chromosomal gene transfer has been noted from SBW25. Indigenous extra-chromosomal DNA such as plasmids have not been found in SBW25. SBW25 has been shown to pick up plasmids from indigenous microorganisms and may therefore act as a vector.

(b) from other organisms to the GMO:

SBW25 has been shown to pick up plasmids from indigenous microorganisms. Selective plating will be done on mercury and potassium tellurite to assess the risk of plasmid uptake into the SBW25:*tgl* cells from indigenous microorganisms. Mercury resistance is a common plasmid-encoded trait.

(c) likely consequences of gene transfer:

Horizontal gene transfer from the GMM to other organisms would interfere with the monitoring process. However the new genes, the cassette containing *tel/gfp/luxAB*, are stably inserted into the chromosome. Chromosomal gene transfer is considered as very unlikely in SBW25 in the absence of selection pressure. No perceivable selection pressure other than tellurite can be envisioned. The introduced genes facilitate the monitoring of the organism, they are stable and, if transfer *should* occur, does not provide a functional advantage to any microorganism.

8. Give references to relevant results (if available) from studies of the behaviour and characteristics of the GMO and its ecological impact carried out in stimulated natural environments (e.g. microcosms, etc.):

There are no studies of ecological impact on SBW25:tgl available yet. There are however studies of other GM-variants of this strain. The variants are considered to be highly comparable.

Maraha, N., A. Backman, and J.K. Jansson, *Monitoring physiological status of GFP-tagged Pseudomonas fluorescens SBW25 under different nutrient conditions and in soil by flow cytometry.* FEMS Microb Ecol, 2004. **51**: p. 123-132.

Unge, A., et al., *Simultaneous monitoring of cell number and metabolic activity of specific bacterial populations with a dual gfp-luxAB marker system.* Appl Environ Microbiol, 1999. **65**(2): p. 813-821.

Unge, A. and J.K. Jansson, *Monitoring population size, activity, and distribution of gfp-luxAB-tagged Pseudomonas fluorescens SBW25 during colonization of wheat.* Microb Ecol, 2001. **41**: p. 290-300

Leij, F.A.A.M.D., et al., *Effect of a genetically modified Pseudomonas aureofaciens on indigenous microbial populations of wheat.* FEMS Microb Ecol, 1994. **13**: p. 249-258.

Lilley, A.K. and M.J. Bailey, *Impact of plasmid pQBR103 acquisition and carriage on the phytosphere fitness of Pseudomonas fluorescens SBW25: burden and benefit.* Appl Environ Microbiol, 1997. **63**(4): p. 1584-1587.

9. Possible environmentally significant interactions with biogeochemical processes (if different from the recipient or parental organism)

The SBW25*:tgl* will interact with other organisms to the same extent as the wild type, or other indigenous fluorescent pseudomonads.

H. Information relating to monitoring

1. Methods for monitoring the GMOs

The resistance to potassium tellurite gives a way of quantifying the amount of SBW25:*tgl* cells by plating on agar plates containing potassium tellurite. Tel^R-positive bacterial colonies appears as black colonies due to the conversion of TeO_2^{2-} to metallic tellurium. Resistance to tellurite has been developed as a way of avoiding introduced antibiotic resistance genes in environmental field trials.

Flow cytometry will be used to quantify the number of gfp-tagged cells in soil and plant samples. It measures the characteristics of single cells in a flowing stream as they pass a series of detectors. Scattered and emitted fluorescent light are collected by two lenses (one set in front of the light source and one at right angles) and by a series of optics, beam splitters and filters to allow specific bands of fluorescence to be measured. Cells are counted and biophysical properties can be measured at rates of more than 1000 cells per second. This allows fluorescent cells to be easily detected in complex environmental samples.
Flow cytometry has been used to monitor SBW25:*gfp/lux* in soil samples.

Fluorescence stereomicroscopy is used to visualize fluorescent bacteria by eye under blue-light illumination as micro-colonies on plant material and on agar plates.

Epifluorescence microscopy is used to study gfp-tagged cells with a magnification up to 1000 X.

Confocal scanning laser microscopy is an imaging technique based on fluorescence that provides greater resolution than standard fluorescence microscopy. This is because of its point illumination and detection properties. A pinhole in front of the fluorescence detector provides the point detection and lasers are used to achieve point illumination. CSLM can be used for detection and localization of *gfp*-tagged bacteria in situ. An important application of CSLM is to visualize cells on the surfaces of and inside plants. CSLM scans one focus layer at a time and if these layers are saved, software can be used to create three-dimensional images of the plant tissues thus displaying the *gfp*-tagged bacteria inside or on the surface of plants.

Luminometry is used to measure luciferase activity from metabolically active cells. The phenotype of the *luxAB* genes, encoding luciferase, is light production, bioluminescence. Bioluminescence is quantified in a luminometer. Detectors measure the emitted light and light intensity is proportional to the number of cells in the sample and their metabolic activity.

Selective plating will be done on mercury and potassium tellurite to assess the risk of plasmid uptake into the SBW25:*tgl* cells from indigenous microorganisms. Mercury resistance is a common plasmid-encoded trait.

2. Methods for monitoring ecosystem effects

The field trial will evaluate the possible risks of introducing a biological control agent into an agricultural field. Potential effects on indigenous bacterial and fungal populations will be evaluated regarding negative ecological effects.

Active microbial cells in soil and on plants will be tagged with *Bromodeoxyuridine,* a thymidine analogue, and the active DNA will be extracted which will correspond to the active microbes in the environment and analyzed by T-RFLP.

T-RFLP (Terminal Restriction Fragment Length Polymorphism) is a powerful tool for assessing the diversity of complex bacterial communities. The method is based on comparison of the different lengths of 16S rRNA fragments. These fragments are amplified by means of PCR with universal primers. One of the primers is labeled with a fluorescent marker in order for the sizes of the restriction fragments to be easily determined and quantified with sequencing. The exact length of the fragments is measured by electrophoresis T-RFLP will be used with general and specific primers for each group, measuring the impact of the inoculum on key functional groups of bacteria.

The impact of the inoculum on denitrification will be estimated using *DGGE* (Denaturing Gradient Gel Electrophoresis) and conserved primers for the functional gene *nosZ*. DGGE is a fast gel-based method used to analyze the genetic diversity of the dominant microbial populations in complex environments. In this study it will be used to investigate the changes in population of denitrifiers in soil. The method is based on electrophoresis of PCR-amplified 16 S – DNA fragments in polyacrylamide gels containing a gradient of denaturants. With this method DNA-fragments of the same length but with different base-pair sequences can be separated.

3. Methods for detecting transfer of the donated genetic material from the GMO to other organisms

No such transfer is considered likely. Chromosomal gene transfer is extremely rare in the environment in the absence of selection pressure and there is no selection pressure for the transfer expected.

4. Size of the monitoring area (m^2)

94.5 m^2

5. Duration of the monitoring

August 2005 – Summer 2006

6. Frequency of the monitoring

The monitoring will last under the growing season August 2005 – Summer 2006. Samples will be taken once or twice a month as long as the soil can be handled. No samples will be taken during the winter season. The monitoring will start again in Spring 2006 as soon as the ground thaws.

I. Information on post-release and waste treatment

1. Post-release treatment of the site

As the recombinant will persist as a stable but minor component, typical of the *P. fluorescens* population (i.e., it exhibits no selective advantage after this period of monitoring) no decontamination will take place. Usually the SBW25 cells do not survive the winter in large numbers.

2. Post-release treatment of the GMOs

Plants will be collected by hand at the end of the experiment, bagged and incinerated. Data collected from the 1993 field release indicates that the GMM does not persist in bulk soil.
Small quantities of waste material (e.g. gloves, soil and plant samples) will be autoclaved after use.

3. (a) Type and amount of waste generated

Plant material colonized with SBW25:*tgl*, samples from the phytosphere and surrounding soil containing recombinants, gloves and boots ´contaminated´ with recombinants. Per sampling occasion several kg of soil, and plant material containing recombinants will be collected. Processing the samples will generate dilution series, enrichment broths (several litres) and agar plates and wash water (> 100 l), all containing recombinants.

(b) Treatment of waste

Excess sample material, dilution series, agar plates, wash water, enrichment broth etc. will be autoclaved.

J. Information on emergency response plans

1. Methods and procedures for controlling the dissemination of the GMO(s) in case of unexpected spread

The plants at the release site will be harvested by hand, bagged and incinerated. The site will be maintained free of vegetation by weeding and herbicide treatment until recombinant cannot be detected (1 cfu/g). Access to the site will be restricted for at least a month after decontamination.

2. Methods for removal of the GMO(s) of the areas potentially affected

The organism is sensitive to a variety of antibiotics (see table 1.) and to chlorine. It is also sensitive to predation by other microorganisms.

Table 1. Antibiotic susceptibility of SBW25:*tgl*. S = sensitive, I = intermediary, R= resistant

Antibioticname	S/I/R	Type of antibiotic
Meticillin	R	Beta lactam
Ceftazidime	S	Cephalosporin
Meropenem	I	Carbapenem
Imipenem	R	Carbapenem
Amikacin	S	Aminoglycoside
Ampicillin	R	Aminopenicillin
Kanamycin	S	Aminoglycoside
Gentamycin	S	Aminoglycoside
Streptomycin	S	Aminoglycoside
Ciprofloxacin	S	Fluoroquinolone
Clarithromycin	R	Macrolide
Sulfonamide	S	Sulfa
Mupirocin	R	Production of some *P. fluorescens*
Tetracycline	S	Tetracycline

3. Methods for disposal or sanitation of plants, animals, soils, etc. that could be exposed during or after the spread

The plants at the release site will be harvested by hand, bagged and incinerated. The site will be maintained free of vegetation by weeding and herbicide treatment until recombinant cannot be detected (1 cfu/g). Access to the site will be restricted for at least a month after decontamination.

4. Plans for protecting human health and the environment in the event of an undesirable effect

No undesirable effect on human health or the environment is thought possible. In the case that the organism is much fitter than indigenous *P. fluorescens* populations, the environment will be protected as outlined under J-2.