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	England
(b)	Notification number	B/GB/04/R39/1
(c)	Date of acknowledgement of notification	03/02/2004
(d)	Title of the project	Strategies for risk assessment, evaluating the environmental impact of fungal diseases suppressing GM bacteria on non-target species
(e)	Proposed period of release	From 01/04/2004 until 01/10/2004

2. Notifier

Name of institution or company: Natural Environment Research Council,
Centre of Ecology and Hyrology Oxford,
Molecular Microbial Ecology Research Laboratory,
Virology and Environmental Microbiology,
Mansfield Road,
Oxford, OX1 3SR.

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 the wild type (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, confirmed the genetic stability of the recipient. These events did not affect stability of the recipient genetically modified inocula. Although similar transfer events were not recorded in 1993 in wheat, we will assay for the acquisition of large plasmids in the proposed release for 2004. This is an advantage of the use of inocula carrying antibiotic resistance, they are readily isolated from the background, high density natural community. These experiment, subsidiary to the purpose of the main release, will provide further insight to the ecology and distribution of the horizontal gene pool. Again we do not anticipate variation in genetic stability in respect of the construct to be released in 2004. This has been chromosomally marked using a disarmed transposon. This produces genetically stable mutants. There is a wealth of data confirming the suitability of disarmed transposons, mini-Tn5, 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.
Spread of the recombinant will not result in selective multiplication in the environment. Cells that disperse might establish only as a proportion of natural microbial populations equivalent to that of normally dispersed SBW25. By using marked organisms this aspect of microbial ecology will be specifically addressed. The recombinant is expected to behave identically to the wild type and no competitive advantage is expected. The released organism is a biological control agent against the fungal phytopathogen *Pythium*. We do not intend to

release any of the pathogen. However, the *P. fluorescens* strain is a phytosphere coloniser and is expected to colonise the leaves and roots of wheat. The recombinant has no known ill effect on the health of the target plant (wheat) It is unlikely that the introduction of this organism into the field has a significant effect on the population dynamics of the indigenous microflora. But this is the issue that the release is intended to address. No impact on other biota is anticipated through natural competition. Only predicted impact considered will be on closely related species with which they will compete for available niches. SBW25 is non-phytopathogenic to wheat and unrelated to any known plant pathogenic bacteria. No impact on beneficial mycorrhizal fungi, no effect of PCA on microbial diversity of the soil and phyllosphere.

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

1. Recipient or parental organism characterisation:

(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 (x) No (.) Not known (.)

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

(i) Yes (.)

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

Atlantic	..
Mediteranean	..
Boreal	..
Alpine	..
Continental	..
Macaronesian	..

(ii) No (x)

(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

Selective plating

Pseudomonas spp. can be isolated on a range of selective media. Selection is based on ability to resist certain antibiotics, e.g., CFC (centrimide, fusaric acid, cephloradine). These media are useful for the isolation of SBW25 and other fluorescent pseudomonads. The Pseudomonad colonies that develop on these iron deficient media produce fluorescent pigments (siderophores) that can be visualised when colonies are exposed to U.V. light. The inocula can also be isolated on media containing rifampicin. The PCA producing variant can also be detected on kanamycin containing media and produces green PCA crystals inside the colonies when grown on LB agar with 2% glucose added.

(b) Identification techniques

Molecular based methods

Identification of the bacteria can be obtained using PCR based methods; we can specifically identify *P. fluorescens* SBW25 using 16S primers which can be confirmed on DGGE gels or

by tRFLP on the sequencing machine. PCR primers can also be used against the specific inserted genes.

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

Yes (.) No (x)

If yes, specify

…

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 (.)

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 is not a known plant pathogen or recognised vertebrate pathogen. After seed inoculation of a variety of plants, SBW25 was detected on sampled leaves and roots.

<u>The Toxicity of phenazine compounds</u>

Naturally occurring phenazines encompass a group of water-soluble nitrogen-containing aromatic pigments produced by members of several bacterial genera including *Pseudomonas, Erwinia, Burkholderia, Brevibacterium* and *Streptomyces* (Giddens *et al.,* 2002 ; Smirnov & Kiprianova, 1990 ; Turner & Messenger, 1986). Almost all phenazines exhibit broad-spectrum activity against Gram-positive and Gram-negative bacteria as well as fungi and higher animal and plant tissues (Smirnov & Kiprianova, 1990). These antibiotic properties are thought to be a consequence of the ability of phenazine compounds to undergo oxidation-reduction transformations and to act as electron acceptors that interfere with the functioning of the respiratory chain (Hassan & Fridovich, 1980). Phenazine compounds produced by root-associated fluorescent *Pseudomonas* spp. function in the suppression of soil-borne plant pathogens (Pierson & Thomashow, 1992; Thomashow & Weller, 1988).

Recent studies of *Pseudomonas aeruginosa* PA14 a human pathogen have demonstrated that the production of the phenazine pigment pyocyanin (1-hydroxy-5-methyl phenazine) contributes to virulence in certain plants and higher animals as well as for effective nematode killing (Mahajan-Miklos *et al.,* 1999; Rahme *et al.,* 1997). In the example of the nematode killing, the study was carried out on the nematode *Caenorhabditis elegans*, when this nematode was fed on lawns of PA14 on specific medium the nematodes died. The role of phenazines in the pathogenesis was also examined in *Aradopsis* and mice. Two independent mutants with disruptions in the *phz*B gene had reduced pathogenicity in both the leaf infiltration model and mouse

burn model. Suggesting that phenazines are multihost pathogenicity factors. Although these findings are significant, despite intensive *in vitro* analyses of phenazines, the physiological significance of their production and their role in *P. aeroginosa* infections in mammals remains controversial. Further studies need to be conducted in order to dissect the biological roles of individual phenazines. These observations suggest that phenazines act as broad-spectrum antibiotics and virulence factors that may contribute directly to the survival of phenazine-producing strains in natural habitats. The expression of these compounds is clearly advantageous to these organisms.

We have not directly undertaken equivalent toxicity tests with SBW25 or its phenazine -1- carboxylic acid (PCA) producing variant, 23.10. However we have not observed any differences in colonisation patterns when plants are inoculated with 23.10 or SBW25, nor have we observed any decrease in plant biomass or observed the development of lesions or symptoms. In fact PCA production by 23.10 increases germination rates in plant seedlings when directly compared to SBW25.

It is important to note that the phenazines represent a complex series of compounds with different forms exhibiting different levels of toxicity to different groups of organisms. The phenazine compound produced by PA14 is different to the compound produced by 23.10 and is produced in a pathogenic bacteria which is very different to SBW25. Furthermore phenazines are natural products and expressed by a number of widely distributed and abundant microorganisms. As these organisms are typical of many soils, and particularly photosphere soils, we do not consider that the proposed release of *P. fluorescens* SBW25 modified to express phenazine -1- carboxylic acid derived from *P. fluorescens* 2-79, constitutes an unacceptable risk to the environment. SBW25 and 2-79 (source of PCA operon) are not pathogenic to plants or animals as they are non-invasive, SBW25 cannot replicate at 37^{o}C.

The isolate 2-79 is typical of many fluorescent pseudomonads associated with take-all suppressive soils. In suppressive soils biotic factors accumulate that result in dramatic reduction in the susceptibility of plants to a number of plant pathogens, typically fungi. The completive accumulation of fluorescent bacteria able to express phenazine like compounds or DAPG has been well documented and of clear advantage to farmers.

The objective of the proposed release is specifically to establish whether the ability of SBW25 to express PCA has an adverse environmental impact. Field experiments are necessary to establish whether non-target soil and plant associated microbiota are affected in their ecology and function.

In field release and extensive mesocosm experiments we have established that SBW25 does not have a negative impact, and can be classified as a plant growth promoting rhizobacteria. In detailed mesocosm experiments conducted on wheat, pea and sugar beet in clay soils, potting composts and commercially available horticultural soils we have not recorded change to soil function or microbial diversity as a result of applying the phenazine producing variant of SBW25, 23.10. In developing the recombinant 23.10 a main objective was that it was of equivalent fitness to SBW25 and that the only observable differences were the ability to express phenazine -1- carboxylic acid and kanamycin resistance. In all colonisation and competition experiments in soils and in the presence of plants both organisms performed the same. The differences observed were in the enhanced ability of 23.10 to protect plants from fungal pathogen attack.

The lack of apparent adverse effects on soil ecosystems was also found in a related study conducted in The Netherlands. The investigators used the identical mini-Tn*5* cassette to modify another soil bacterium *Pseudomonas putida* WCS358r to generate the PCA producing variant WCS358::phz. These were applied to field grown wheat seeds and the ecology of the inoculum and its impact on soil diversity and function monitored using methods similar to those we describe. The same organism was also released in a study in the USA and compared to a variant, WCS358::*phl* that had been similarly modified to express DAPG rather than PCA. In both experiments the *de novo* expression PCA had no lasting impact.

In summary:

- Phenazine -1- carboxylic acid (PCA) is a natural compound produced by bacteria typical of many soil habitats.
- The development of Take-all suppressive soils which have been recorded at Rothamsted Research, are probably due to the competitive selection of rhizobacteria able to express antifungal compounds such as a phenazine.
- The related studies with PGPR bacteria modified with the same PCA producing mini-Tn*5* in The Netherlands and USA had no adverse impact.
- The objective of the proposed release is to test whether a typical plant associated bacterium such as SBW25 modified to express a biologically functional trait does cause an impact, or whether the natural resilience of soils is an effective buffer. This information is necessary as a number of genetically modified biological control agents are under consideration.
- In our assessment the impact or likelihood of environmental harm resulting from the proposed release of 23.10 is negligible.

8. Information concerning reproduction

(a) Generation time in natural ecosystems:
The generation time in natural ecosystems is difficult to assess and depends on food supply, pH, inhibiting substances, water potential, competition and temperature. The organism multiplies by cell division and there is no sexual reproductive cycle. Mean generation time of SBW25 at 28^{o}C in LB broth determined at ca. 1.0 h. *In situ* estimates of growth rate have been extrapolated from glasshouse and field grown inoculated plants. Maximal rates for SBW25 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 time in natural ecosystems is difficult to assess and depends on food supply, pH, inhibiting substances, water potential, competition and temperature. *In situ* estimates of growth rate have been extrapolated from glasshouse and field grown inoculated plants. Maximal rates for SBW25 mean generation time on the plant surface are assumed to be in the order of 2-5h.

(c) Way of reproduction: Sexual .. Asexual x

(c) Factors affecting reproduction:
Food supply, pH, inhibiting substances, water potential, competition and temperature.

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	None

(b) relevant factors affecting survivability:
Pseudomonas spp. are not known to produce recognised long term survival structures. However pseudomonads are polymorphic and able to tolerate adverse environmental conditions. SBW25 does not survive after removal of plants and plant material, it is also cold intolerant and has been shown not to survive through the winter frosts. An objective here is to establish whether the variant of SBW25 carrying the operon for PCA biosynthesis changes persistence in the field. Under recent laboratory and mesocosm investigations we have not observed any change in the ecology of this variant when compared to wild type SBW25 in uninfected plants. In microcosm experiments conducted on pea, wheat and sugar beet in the presence of the phytopathogen *Pythium ultimum* var. Trow., the causative agent of damping-off disease of seedlings, the PCA producing variant established greater population densities and biocontrol efficacy. This improvement in "fitness" was associated with pathogen control and improved plant health

10. (a) Ways of dissemination
Dispersal by wind and water is thought to be limited since cells produce sticky mucilage that helps in attaching the organism to (plant) surfaces. Active dispersal over short distances in water films is possible since each cell has flagella that can be used for dispersal.
There is a potential for short distance dispersal by rain splash, particularly from leaves and possibly from the soil, but distances involved are likely to be small. The implementation of the 1 m deep guard rows 1 m away from the plots around the whole release site will protect the outer area of the site from possible rain splash. If this occurs we will be able to detect it when we sample the guard rows and surrounding areas. This will provide important data to us about the dispersal of this organism.
Long distance spread is possible in that the organism can attach itself to organisms that feed on, or pass through the crop. Both vertebrates and invertebrates can act as potential dispersal agents. Studies on sugar beet demonstrate that flying insects can be contaminated with GMMO after landing on colonised leaves. Studies by us also demonstrated that insects play a role in the dispersal of *Pseudomonas* spp. It is, therefore, reasonable to assume that any organism (birds, mammals, etc.) that enters the crop can act as a vector of the recombinant (or any other micro-organism). However, it is unlikely that such dispersal can result in the colonisation and propagation of viable populations of transferred bacteria. Only high densities of phytophagous caterpillar, *Mammestra brassica*, resulted in the effective dispersal of colonising populations of SBW25 in the phyllosphere. Transfer to the rhizosphere was not recorded. Transfer by the caterpillars was from plants colonised by SBW25

after seed inoculation. Third instar larvae were added in netted enclosures to established, large plants colonised by SBW25. These plants were surrounded at normal planting densities with uninoculated sugar beet plants of the same age and size, with their leaves touching. The caterpillars only moved from the plants to which they were added when it had effectively been defoliated. As they migrated they carried bacteria, at the new resource of uninoculated plants the bacteria were deposited in the growing leaf bud and colonised to normal densities within 2-3 weeks as those recorded following original seed inoculation. Therefore the caterpillars transferred bacterial populations, we assume that the high densities of caterpillars and therefore the high quantities of frass produced provided additional nutrients that benefited the population expansion of SBW25. This was also enhanced as the caterpillars tended to rest at the heart of the sugar beet plants, where the new shoots develop and where SBW25 can establish. SBW25 did not establish on larger expanding or mature leaves exposed to the caterpillars. However please note that SBW25 is a poor coloniser of the wheat phyllosphere.

(b) Factors affecting dissemination

Multiplication is governed by at least five factors:

a) Temperature: Multiplication occurs at temperatures greater than 4^{O}C. At temperatures of 37^{O}C and above no growth takes place and the organism will die within four days. Optimum growth conditions are between 25 and 30^{O}C.
At temperatures below freezing the bacterium is killed.
b) Nutrition: The isolate is thought to thrive on plant exudates from a wide variety of plants and easily degradable carbon sources.
c) Humidity: Although the isolate can to some extent survive dry conditions (RH 60%), multiplication only takes place when enough moisture is available (RH 100%).
d) Competition, predation and parasitism: The exact mechanisms of how these factors influence the population dynamics of *P. fluorescens* SBW25 are not known, but experiments have shown that the recombinant or wild type cannot survive on decaying wheat roots at 100% RH at 25^{O}C.
e) pH: The isolate thrives under neutral to slightly alkali conditions.

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)
The recombinant SBW25EZY-6KX was introduced into the open environment in 1993. Two parallel investigations were undertaken. One at IVEM where bacteria were introduced as a seed dressing to sugar beet and the other at HRI where bacteria were introduced as a seed dressing and as a spray application to developing wheat. (consent for release; B/93/R3/1, B/94/R3/3) .

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
For the bacterium to be resistant to kanamycin and constitutively express phenazine-1-carboxylic acid.

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 on mini-Tn*5*Km1 with the PCA biosynthesis genes added to produce pUTkm-*phz*

(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 (Kanamycin resistance)
other, specify …

Indication of which antibiotic resistance gene is inserted
Km^r determinant of Tn*903* (nptI, APH(3')I)

(e) Constituent fragments of the vector

Kanamycin resistance: Km^r determinant of Tn*903* (nptI, APH(3')I), was excised as a 1.7 kb *Bam*H1 fragment from the mini-Tn*10Km* of phage λ1105 (Way *et al.,* 1984), and inserted in to pUC18Sfi before introduction in to pUT to produce mini-Tn*5Km1*. pUT was derived from pGP704, a 1.5 Kb *Sal*I fragment from pTet containing Tn*5 tnp* devoid of *Not*I sites (*tnp**) was inserted into the single *Sal*I site of pGP704. The vector also contains the *bla* gene (which confers resistance to a variety of β-lactam

antibiotics including ampicillin and piperacillin, the R6K (ori R6K) origin of replication and the RP4 *ori*T (mobRP4) genes.

Phenazine-1-carboxylic acid (PCA)

The PCA biosynthetic operon *phzABCDEFG* is from *P. fluorescens* 2-79 (Thomashow and Weller 1998), cloned previously as pT7-6A-G, was excised as a 6.7kb *Bgl*II-*Xba*I DNA fragment and ligated into the *Bam*HI and *Xba*I sites of cloning vector pALTER-*Ex*1distal to a tac promoter (which means the PCA will be expressed constitutively). This 6.7 kb fragments was then cloned into the *Sfi*I site of mini-Tn*5*Km1 yielding pUTkm-*phz*.

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

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

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

The PCA biosynthetic operon *phzABCDEFG* is from *P. fluorescens* 2-79. Km^r determinant of Tn*903* (nptI, APH(3')I)

(b) Source of each constituent part of the insert

The PCA biosynthetic operon *phzABCDEFG* is from *P. fluorescens* 2-79. Km^r determinant of Tn*903* (nptI, APH(3')I)

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

Kanamycin resistance and PCA biosynthesis

(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. Information on the organism(s) from which the insert is derived

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

(i)	order and/or higher taxon (for animals)	bacterium
(ii)	family name for plants	…
(iii)	genus	Pseudomonas
(iv)	species	fluorescens
(v)	subspecies	…
(vi)	strain	2-79
(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 (.) No (x) Not known (.)
If yes, specify the following:

(b) 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 (.)

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 23.10 (GM variant of SBW25)is any more or less genetically stable than other fluorescent pseudomonads. By FAME-MIS analysis populations of SWB25, inoculated onto glasshouse plants, demonstrate no distinguishable variation. Data obtained by repeated sub-culture of the recombinant, in non-selective broth, confirmed the genetic stability of the recipient. Natural acquisition of plasmids from the indigenous phytosphere community did not affect stability of the recipient genetically modified inocula. Although similar transfer events were not recorded in 1993 in wheat, we will assay for the acquisition of large plasmids in the proposed release for 2004. This is an advantage of the use of inocula carrying antibiotic resistance; they are readily isolated from

the background, high density natural community. This experiment, subsidiary to the purpose of the main release, will provide further insight to the ecology and distribution of the horizontal gene pool. Again we do not anticipate variation in genetic stability in respect of the construct to be released in 2004. This has been chromosomally marked using a disarmed transposon. Which produces genetically stable mutants. There is a wealth of data confirming the suitability of disarmed transposons, mini-Tn*5*, for marking Gram-negative bacteria.

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

Yes (.) No (x) Unknown (.)

(a) to which of the following organisms?

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

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

…

4. Description of identification and detection methods

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

Selective plating

Pseudomonas spp. can be isolated on a range of selective media. Selection is based on ability to resist certain antibiotics, e.g., CFC (centrimide, fusaric acid, cephloradine). These media are useful for the isolation of SBW25 and other fluorescent pseudomonads. The Pseudomonad colonies that develop on these iron deficient media produce fluorescent pigments (siderophores) that can be visualised when colonies are exposed to U.V. light. The inocula can also be isolated on media containing rifampicin. The PCA producing variant can also be detected on kanamycin containing media and produces green PCA crystals inside the colonies when grown on LB agar with 2% glucose added.

Molecular based methods

Identification of the bacteria can be obtained using PCR based methods; we can specifically identify *P. fluorescens* SBW25 using 16S primers which can be confirmed on DGGE gels or by tRFLP on the sequencing machine. PCR primers can also be used against the specific inserted genes.

(b) Techniques used to identify the GMO

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F. Information relating to the release

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

A single release site has been selected for investigations at Rothamsted Research. Seed will be inoculated with the recombinant organism or with the wild type organism.

Objectives of the release

This release is part of a proposal directed to Roame A (RG01) to inform DEFRA of any potential impact or harm that may arise from the use and application of genetically modified bacteria released to the environment to provide plant protection and plant growth promotion by the *de novo* expression of antifungal compounds. The principal objectives are to extend our current approaches for impact assessments to include field releases of a genetically modified bacterium. This proposal builds on a substantial understanding of the genetics and biology of a rhizosphere competent fluorescent pseudomonad, *Pseudomonas fluorescens* SBW25 that we have modified to express an additional antifungal compound (AFC), phenazine-1-carboxylic acid (PCA). This genetically modified biological control agent (23.10) protects seedlings from 100 x the normal challenge of *Pythium ultimum* (the causative agent of damping-off disease) when compared to either the wild type *P. fluorescens* SBW25 (the recipient of the genes) or *P. fluorescens* 2-79 (the original source of the PCA biosynthetic pathway, *phz*ABCDEFG). We will determine whether the expression of PCA directly influences the ecosystem function and diversity of non-target, plant beneficial rhizosphere bacteria and mycorrhizal fungi in spring wheat. The output from this project will provide input to DEFRA policy "*to provide scientific data .. to establish whether any GMMO released would pose any discernible risk of harm to the agricultural environment ...* and *based on sound scientific principles .. affirm the adequacy of risk assessment..*" that might result from the future environmental release of GMMs and GMO plants with commercial potential for plant protection and plant growth promotion. The purpose of this release is not to assess biocontrol efficacy of the inocula but to evaluate whether the use of a naturally occurring bacterium modified to express a functional product, also derived from a closely related naturally occurring bacterium, impacts on the ecology of soil/plant habitats.

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 trial will be conducted at Rothamsted Research which is an experimental farm located near Harpenden, Hertfordshire. The one grid square that best locates the release is TL 120 136.

(b) Size of the site (m^2): … m^2
(i) actual release area (m^2): 12 m^2
(ii) release site (m^2): 21 x 13.5 m (283.5 m^2)

(c) Proximity to internationally recognised biotopes or protected areas (including drinking water reservoirs), which could be affected:
The site is bordered on three sides (E, W, N,) by (for at least 500 m) by arable agriculturable land. The crops surrounding the site during the first season will be Spring Barley. The fourth side (S) is bordered by a flat concrete area approximately 20 m from farm buildingscontaining offices (see map, figure 5). Other farm buildings lie further to the south of the site (for about 100m). The land south of the farm buildings is used for arable agriculture. Two residential dwellings (semi-detached) lie approximately 100 m to the east of the site. The nearest other residential land lies more than 500 m north-east of the site. There are areas of woodland approximately 600 m to the north-west, 450 m to the south-west and 500 m to the south-east of the site. There are no specific protected areas of relevance immediately adjacent to the site. A borehole for irrigation water is situated 80 m from the site

(d) Flora and fauna including crops, livestock and migratory species which may potentially interact with the GMO
The plot is surrounded by a gated high fence to safeguard the crop against large herbivores and to which access will be limited to the approved members of staff. A monitoring strategy has been devised to measure the natural dispersal of the GMO.

Insects and birds

4. Method and amount of release

(a) Quantities of GMOs to be released:
Current investigations confirm that the application of the PCA producing variant of SBW25 (23.10) inocula as a soil drench to provide a dressing (10^6 cfu/g of soil) coincident with the planting of seeds in microcosms and mesocosms. This method provides effective colonisation of the plant rhizosphere. For the field a single inoculum of 10^6cfu/g of soil will be applied as a soil drench to the approximate top 10 cms of soil. Overnight cultures of bacteria grow to 10^8 cfu/ml, culture will be washed twice in sterile distilled water and resupended in their original volume. In total 12 m^2 will be planted with 360 seeds per 1 m^2 and treated with the GMMO. Each plot of 4 m^2 (2x2 m) will be inoculated with 250 ml of washed culture containing 10^8 cfu/ml. The drench will be applied evenly using a watering can. (After use which will be labelled, double bagged and returned to Oxford for decontamination.) Therefore approx. 1.2 x 10^{11} recombinant bacteria will be introduced to the entire area of GM plots.

(b) Duration of the operation:

The release will take place from March/April 2004 until August/September 2004. Post harvest monitoring will take place for 2 further years.

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

The plot is surrounded by a gated high fence to safeguard the crop against large herbivores and to which access will be limited to the approved members of staff. A monitoring strategy has been devised to measure the natural dispersal of the GMO. Precautions will be taken to avoid the spread of recombinant during sampling. Footwear will be disinfected and gloves changed.

5. Short description of average environmental conditions (weather, temperature, etc.)
The site has an average rainfall of just over 700 mm, sunshine hours are about 1500 h, average annual temperature is 9.5 ^{o}C.

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.
No previous releases

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

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)
…

3. Any other potentially significant interactions with other organisms in the environment
This is why the release is to take place to measure significant interactions

4. Is post-release selection such as increased competitiveness, increased invasiveness for the GMO likely to occur?
Yes (.) No (x) Not known (.)
Give details
SBW25 does not survive after removal of plants and plant material, it is also cold intolerant and has been shown not to survive through the winter frosts

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

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

None expected

(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:
unlikely

(b) from other organisms to the GMO:
unlikely

(c) likely consequences of gene transfer:
none

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.):

Summary of soil and plant inoculation mesocosm studies with 23.10

Commercially derived loam top soil (www.gemgardening.co.uk), typical of that used in the glasshouse production of plants was prepared (750 g) in seed trays (22.5 x 16 x 7.5 cm). The soil in half of the available trays was mixed with *Pythium ultimum* inocula (1x 10^3 pfu/g soil). Trays were then surface drench with 150 ml of the suspensions of SBW25 or 23.10 (to provide average inocula density of ca. 3 x 10^6 cfu/g soil); sterile water alone was included as the untreated control. Soils (t=3) were then left to acclimatize for 24 h in a plant growth room (21^{o}C, 18 h photoperiod) before planting with seeds (50 per tray) of pea (*Pisum satvium* var. *quincy*), wheat (*Triticum aestivum* var. *pena wawa*), or sugar beet (*Beta vulgaris* var. *amythyst*). The relative soil moisture content was maintained throughout the experiment by regular application of water to the soil surface.

Three plant species were investigated, pea (*Psium satvium* var. *quincy*), wheat (*Triticum aestivum* var. *pena wawa*), and sugar beet (*Beta vulgaris* var. *amythyst*). These represent typical hosts for damping-off disease, which results in considerable agronomic loss world wide, and are potential commercial targets for the use of plant growth promoting rhizobacteria (PGPR) as biological control agents.

Plants were sampled over several life stages little or no effect was recorded on the density of background communities, as determined by plate count methods, following the introduction of bacterial SBW25 inocula when treatments were compared with the appropriate controls. Total plate counts for aerobic heterotrophs forming colonies were determined using data collected over the whole study, for peas 2.49 x 10^9 CFU/g wet root tissue (n=72, Sd 2.43),

for wheat 6.37 x10^8 CFU/g wet root tissue (n=72, Sd 6.66) and for sugar beet 3.39 x 10^9 CFU/g wet root tissue (n=54, Sd 2.43). SBW25 and 23.10 inocula established higher population densities in the roots of peas (SBW25 2.1 x10^9 CFU/g wet root tissue and 23.10, 2.04 x10^9 CFU/g wet root tissue) and wheat (SBW25 9.12 x10^7 CFU/g wet root tissue and 23.10 4.3 x10^7 CFU/g wet root tissue) when compared to sugar beet seedling (SBW25 1.58 x10^7 CFU/g wet root tissue and 23.10 6.7 x10^6 CFU/g wet root tissue). Although densities of SBW25 when compared with 23.10 were not significantly different in each plant type over the 5-6 weeks of the study. All data collected from plate counts was analysed using single factor ANOVA to establish if there were any significant differences over time and within and between the treatments. Fungal counts were significantly lower in SBW25 and 23.10 treatments where infection was introduced (sugar beet 1.8 x10^5 PFU/g wet root tissue; infected sugar beet, 1.07 x10^5 PFU/g wet root tissue; SBW25 treated sugar beet and 1.12 x10^5 PFU/g wet root tissue; 23.10 treated sugar beet, p=0.39. Wheat 1.9 x10^5 PFU/g wet root tissue; infected wheat, 1.07 x10^5 PFU/g wet root tissue; SBW25 treated wheat and 1.12 x10^5 PFU/g wet root tissue; 23.10 treated wheat, p=0.39).

Impacts on total population dynamics were measured using complementary methods, including plate counts, 16S/18S PCR-DGGE molecular community diversity profiling. Direct physiological measures of perturbance to AM root association to provide an indicator in established mixed plant standings, typically agricultural/field margin pasture. Our detailed multifactiral approach showed that impact was only transient in line with the studies of others. The recorded changes in diversity and community succession correlated with the plant species, time of sampling and growth stage of the plant rather than the use of SBW25 or 23.10 bacterial inocula. Culture independant molecular fingerprinting analysis monitored changes in bacterial and fungal diversity over time following introduction of either the wild type SBW25 or 23.10. Surprisingly few differences were observed in profiles produced by RT-PCR of 16S rRNA or PCR of 16S DNA. Principal component analysis of within gel diversity for each plant type were produced for the root and shoot for both bacterial and fungal diversity. In all cases these profiles revealed no major impact on diversity due to incoula, and that diversity, community structure and successsion was directly determined by plant type and the presence or absence of disease or bacterial inocula's ability to control damping-off further than any direct impact of the bacterial inocula.

The impact of disease on diversity and function is lessened in the presence of inocula. Although no significant shifts were recorded when wild type or 23.10 inoculated plants were compared the combination of inocula and disease, did result in a discernible shift in community banding patterns when compared to untreated controls or bulk soil. Pea, wheat and sugar beet each influenced and were colonized by different communities selected from the soil. The impact of the plant type and infection was greater than the impact of inocula in all assessments made of diversity and activity. Following complete analysis of data from hundreds of samples taken from replicated microcosms we can conclude that the methods applied did not reveal any adverse effects due to the use of bacterial inocula or the *de novo* synthesis of the antifungal compound PCA by the GM BCA *P. fluorescens* SBW25. No observed increase or decrease in any operational taxonomic unit (OTU) band was observed within plant type comparisons. From this data we conclude that no major groups were excluded or enriched as a result of BCA and GM BCA treatment.
We successfully studied the histology of root AM infection/colonisation to determine the impact of an antifungal compound producing fluorescent pseudomonad. AM infection was abundant in plant species typical of a grassland field margin pasture. Percentage colonisation data were analysed using single factor ANOVA, values were compared between treatments

and with time. A small decrease in mycorrhizal association was recorded in wild type or 23.10 treated samples when compared with the water control 4 days after inoculation although this decrease was not significant p=0.15. However this minor perturbation was only transient and the relative amount of AM association with roots had increased 2-3% 16 days later (no significant difference, p=0.56) confirming that mycorrhizal associations were active and not effected by treatment over the 20 day period (no significant difference, p=0.329).

9. Possible environmentally significant interactions with biogeochemical processes (if different from the recipient or parental organism)
None

H. Information relating to monitoring

1. Methods for monitoring the GMOs
Selective plating

Pseudomonas spp. can be isolated on a range of selective media. Selection is based on ability to resist certain antibiotics, e.g., CFC (centrimide, fusaric acid, cephloradine). These media are useful for the isolation of SBW25 and other fluorescent pseudomonads. The Pseudomonad colonies that develop on these iron deficient media produce fluorescent pigments (siderophores) that can be visualised when colonies are exposed to U.V. light. The inocula can also be isolated on media containing rifampicin. The PCA producing variant can also be detected on kanamycin containing media and produces green PCA crystals inside the colonies when grown on LB agar with 2% glucose added.

Molecular based methods
Identification of the bacteria can be obtained using PCR based methods; we can specifically identify *P. fluorescens* SBW25 using 16S primers which can be confirmed on DGGE gels or by tRFLP on the sequencing machine. PCR primers can also be used against the specific inserted genes.

2. Methods for monitoring ecosystem effects
Rhizosphere sub-samples will be prepared for
- Measurement of PCA concentration.
- Nycodenz fractionation for CTC-FACS separation of the active bacterial component and
- Total nucleic acid extraction for total community profiles (DGGE- 18S and 16S rDNA).
- Plate count methods to monitor the culturable component and fate of inocula.
- Extent of mycorrhizal colonisation
Soil enzyme activity measures.

3. Methods for detecting transfer of the donated genetic material from the GMO to other organisms
It is assumed that if any transfer occurs it will occur between related organisms. Therefore we will plate out on pseudomonad selective agar medium containing kanamycin. We will then check for phenazine production on LB agar containing 2% glucose (green crystals form inside the PCA positive colonies). We can also clarify that these are not rifampicin resistant as well. If transfer has occurred we will broaden our search by looking at all the bacteria isolated on kanamycin plates. In addition although not sensitive enough alternative methods may include the use of PCR.

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

5. Duration of the monitoring
Two years post harvest

6. Frequency of the monitoring
Monthly

I. Information on post-release and waste treatment

1. Post-release treatment of the site
The site will be monitored for up to two years or until the GMO can not be found on three consecutive samples.

2. Post-release treatment of the GMOs
All samples containing the GMO will be safely transported to the lab and autoclaved

3. (a) Type and amount of waste generated
Per sampling occasion some kg of soil and plant material containing recombinants will be collected. All samples will be put into labelled sealed bags, and then put in to a second sealed bag before placing in a sealed metal container. Samples will then be transported to Oxford for processing. Processing the samples will generate dilution series, enrichment broths (several litres) and agar plates (> 1000 per sample occasion) and wash water (> 100 l), all containing recombinants.

3. (b) Treatment of waste
All waste will be autoclaved in the laboratory

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 can not be detected (10 cfu / g). Access to the site will be restricted for at least a month after decontamination. In the event of spread of the bacteria as a result of vandalism, all plant material will be collected, bagged and incinerated. All areas which come in to contact with rhizosphere samples i.e. the released organism will be sampled to check for contamination. As the bacteria does not survive without plant material, we envisage that it will only be found for a short time. These areas will be sampled until the organism is no longer found. If for some reason the released organism appears to persist better than we expect we can solarize (cover with black polyethene) the area to eradicate the organism. If the organism persists far better than we anticipate, the areas of soil still contaminated after two years of monitoring can be removed and decontaminated. If the organism was found out side the fenced area any plants growing within 1 m would be removed and autoclaved as previously described. Contaminated soil areas will be solarized and monitoring would continue. If contamination was not removed the soil will be removed and decontaminated.

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

It is anticipated that the GMO will die out.

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

4. Plans for protecting human health and the environment in the event of an undesirable effect
Samples will be collected and autoclaved