Information relating to ANNEX II

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

1) The parental (host) organism is a member of the *Pseudomonas fluorescens* species of bacteria. It was first characterised by Shanahan *et al.* (1992).

2) Its complete name is *Pseudomonas fluorescens* strain F113. The GMO, *Pseudomonas fluorescens* F113lacZY is based on this wildtype. The strain to be used in the release trial is a spontaneous rifampicin resistant mutant derivative of this GMO. The GMO, *Pseudomonas fluorescens* F113rifpcb, is derived from the parental organism *P. fluorescens* strain F113rif. *Pseudomonas fluorescens* F113rif is a spontaneous rifampicin resistant derivative of the wild type organism *P. fluorescens* F113 (Brazil *et al.*, 1995).

3) Geographical distribution of the organism.

a) The isolation of *P. fluorescens* F113 were reported by Shanahan *et al.* (1992) whereby the organism was isolated from the root hairs of a sugar beet plant and characterised as a fluorescent bacteria, Gram-negative, nonfastidious with a motile rod that is oxidase and catalase positive which indicates that it is a member of the *Pseudomonas* species as outlined in *Bergey's Manual of Determinative Bacteriology* (Bergey, 1982).

b) The bacterial species *P. fluorescens* is an ubiquitous organism in Europe, they are common members of the microflora of soil and water. *P. fluorescens* F113 was first isolated from the rhizosphere of a sugar beet plant taken from soils in Counties Cork and Tipperary, Ireland (Atlantic ecosystem).

The isolation of the strain F113 outside Ireland, including Denmark, has not been reported. However, *P. fluorescens* strains equivalent to F113 can certainly be isolated from plant rhizosphere everywhere, as *P. fluorescens* is a normal inhabitant of plant rhizosphere. In fact other *P. fluorescens* strains with biocontrol abilities have been isolated in various parts of the world.

c) Close relatives of *Pseudomonas fluorescens* strain F113 are regularly used in agriculture worldwide:

- *P. fluorescens* WCS374 has been applied commercially on radish seed as "BioCoat" (S&G Seeds) to suppress fusarium wilt. At present, this product is not available because of its relatively high costs of production, appearence of resistant radish cultivars and because production efficiancy is less than 100%. (Cited from: Plant microbe Interactions and Biological Control, Eds. G.J.Boland & L. David Kuykendall, 1998, Marcel Dekker).
- *P. fluorescens*, commercial name: Dagger G (Ecogen Inc., Langhorne, PA) against Pythium ultimum, Rhizoctonia solanii for cotton seed treatment. (See: ACS Symposium Series 595, Biorational Pest Control Agents, Formulation and Delivery, Eds. F.R.Hall and J.W.Barry, 1995).
- *P. fluorescens* bv5, commercial name: VICTUS (Sylvan, Kattanning, PA) against other Pseudomonads (P. tolaasii) in mushrooms. (See: ACS Symposium Series 595,

Biorational Pest Control Agents, Formulation and Delivery, Eds. F.R.Hall and J.W.Barry, 1995).

- *P. fluorescens*, commercial name: Conqueror (Mauri Foods, Australia), as above against same Pseudomonad in mushrooms. (See: ACS Symposium Series 595, Biorational Pest Control Agents, Formulation and Delivery, Eds. F.R.Hall and J.W.Barry, 1995).
- The website of the U.S. Department of Agriculture, Beltsville, Biocontrol of Plant Diseases Laboratory, http://www.barc.usda.gov/psi/bpdl/bpdlprod/bioprod.html lists and describes products based on live microorganisms on the market. Among them we find 3 products based on *P. fluorescens*: BlightBan A506, Conquer and Victus, and two based on *Burkholderia cepacia*: Intercept and Deny.
- Other *Pseudomonas*-products are presently on the market. Well known is *P. chlororaphis* (commercial name: CEDOMON), which is developed by Gerhardson (Uppsala) and used in Sweden, Norway and Finland, marketed by "BioAgri" and used on wheat (large areas treated with CEDOMON) (US patent 5,900,236 "Composition and method for controlling plant diseases using *Pseudomonas chlororaphis* strain NCIMB 40616).

d) Recently, *P. fluorescens* F113 has been regularly used and kept in laboratory experiments in The National Environmental Research Insititute (NERI), Røskilde, Denmark.

4) Natural habitat of the organism.

a) The microorganism *P. fluorescens* strain F113 is found in the soil in association with plant roots. F113 was first isolated from the rhizosphere of sugarbeet plants (Shanahan *et al.*, 1992)

5) Detection and identification.

a) *P. fluorescens* F113rif is commonly detected by plating soil/root suspensions on plates of the minimal sucrose asparagine agar (SA) (sucrose, 20 g L^{-1}; asparagine, 2 gL^{-1}; K_2HPO_4, 1 g L^{-1}; purified agar, 15 g L^{-1}; with the addition of 2 mM $MgSO_4$ after autoclaving) amended with 50 mg ml^{-1} rifampicin, growing as fluorescent yellow-green colonies after 24 h when grown at 28 ^oC and as brown pigmented colonies after 4 days at 28 ^oC.

b) A PCR based fingerprinting detection system has been designed specifically for the control strain F113lacZYrif. A non-antibiotic marker cassette, mini-Tn5, was inserted into the chromosome of *P. fluorescens* F113 (Fedi *et al.*, 1996) creating a stably integrated *lacZY* cassette, with an unaltered effect in ecological fitness. The insertion of the *lacZY* genes created an unique junction region on the chromosome of the novel strain and this region has been exploited to develop a PCR based fingerprinting detection system. Two primers were designed which will specifically amplify the unique junction region of the mini Tn5 insertion on the chromosome of the control strain F113lacZYrif. This detection system will only generate a PCR product when the DNA of F113lacZYrif is present in the test samples.

In addition, the presence of the *lacZY* genes confers the lactose utilisation phenotype to the strain and therefore the control strain can be detected as blue colonies by culturing

on minimal medium M9 ($Na_2HPO_4.7H_2O$, 12.8 g L^{-1}; KH_2PO_4, 3 g L^{-1}; NaCl, 0.5 g L^{-1}; agar, 15 g L^{-1}; $MgSO_4$, 2 mM, $CaCl_2$, 10 mM) with 0.5 % lactose as a sole carbon source supplemented with 40 mg/L of 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-Gal) and 50 mg ml^{-1} rifampicin.

6) Safety classification.

Under the existing Community rules relating to the protection of human health, the recipient organism is not classified in Denmark or at the EU level.

However, in Germany *Pseudomonas fluorescens* is classified in risk group 1. This classification is given with the following remark: "In invidual cases shown or suspected to be a pathogen, mostly with strongly immune-compromised human; species identification often not reliable." (Berufsgenossenschaft der chemischen Industrie. 1997. Sichere Biotechnologie, Eingruppierung biologischer Agenzien: Bakterien. Merkblatt B 006, 2/97, ZH 1/346. Jedermann-Verlag, Heidelberg).

This means, in Germany *Pseudomonas fluorescens* is positively classified as Group 1 for enclosed (indoor) use, i.e., considered safe with regards to workers' protection at their workplace, even in the face of reports on secondary infections of seriously ill patients.

In other parts of the world, *P. fluorescens* has not been considered for inclusion in the list of classified organisms. According to the information service of the Office of Biosafety Canada (http://www.hc-sc.gc.ca/hpb/lcdc/biosafty/index.html), which offers a list of known risk group classifications worldwide, *P. fluorescens* is not classified in Canada, by the NIH, by the EU, by the state of Belgium, or in Australia, nor anywhere else.

7) Pathogenicity.

In addressing the issue of pathogenicity, the topics to be considered are

- infection of healthy and of immuno-compromised individuals
- toxic effects
- carcinogenic effects
- allergenic effects

Disease-related bacterial properties to be considered include

- adherence to cells
- invasion of cells
- cell damage
- sensitivity to antibiotics

As *Pseudomonas fluorescens* F113 is a documented biocontrol strain, enhancing plant health (Carroll *et al*. 1995; Naseby & Lynch 1999), it is sufficient to consider pathogenicity to humans and animals.

In the available literature, the species *Pseudomonas fluorescens* is not considered pathogenic, or harmful in any other way (including its extracellular products), either

living or dead. In particular, root colonising *Pseudomonas fluorescens* is considered non-pathogenic, non-toxic, non-virulent micro-organism (they are part of the normal flora of many crop plants). There is also no evidence for carcinogenic or allergenic effects.

Pseudomonas fluorescens does not possess any external cell structures facilitating adherence to cells, nor any properties related to the invasion of cells or cell damage. In this context, it is important to note the difference between *P. fluorescens* and fluorescent pseudomonads. The taxonomic group of fluorescent pseudomonads includes *P. aeruginosa*, a Group 2 organism and a known opportunistic pathogen.

The host strain *P. fluorescens* F113 does not harbour any relevant antibiotic resistances, while the two GMOs, *Pseudomonas fluorescens* strains F113rifpcb and F113lacZYrif, are resistent to rifampicin, but sensitive to other antibiotics. These resistances were obtained by spontaneous mutation, with the intention of improving the GMOs' detection in soil.

A search of the available literature addressed the question, whether there was any recent evidence showing pathogenicity of *P. fluorescens* either in humans or animals? In the databases we found two reports:

- One was a report on the isolation (among other pseudomonads) from sick chickens of two haemolytic *P. fluorescens* strains. One of them was totally non-pathogenic to chicks while the other caused illness to 30% of them compared to the 100% rate of several *P. aeruginosa* strains (Avian Dis 1993. Jan-Mar;37(1):6-9. Classification, pathogenicity, and drug susceptibility of haemolytic gram-negative bacteria isolated from sick or dead chickens. Lin MY, Cheng MC, Huang KJ, Tsai WC).

- In the other study (George SE, Nelson GM, Kohan MJ, Brooks LR, Boyd C. 1999. Colonization and clearance of environmental microbial agents upon intranasal exposure of strain C3H/HeJ mice. J Toxicol. Environ. Health-Part A 56(6):419-431), mice were exposed to laboratory strains of *P. fluorescens* and *B. cepacia* . After intranasal exposure at 10^7 cfu per mouse, the lungs were cleared of *P. fluorescens* after 1 d, and from the intestines after 3 h, while *Burkholderia cepacia* persisted in the lungs for over 14 d. In contrast, pulmonary exposure (i.e., an intranasal overdose) was fatal at $6.15*10^8$ cfu/mouse for *P. fluorescens*, and $1.34*10^8$ cfu/mouse for *Burkholderia cepacia*. The lethal effect was not attributed to an infection by *Pseudomonas*, but to a reaction of the mice's tissues to cell surface factors of the bacterial cells. In other words, the findings do not indicate the initiation of a disease by the bacteria, but a toxic effect of the extreme dosis applied. The lethal dosis of *P. fluorescens* calculates at ca. $2.8*10^{10}$ cfu/kg, corresponding to $2*10^{12}$ cfu/adult human (75 kg), employing a simple conversion by bodyweight only. The total number of GMO *P. fluorescens* to be released in the field trial is $6.3*10^{11}$ cfu, the bacteria being mixed in a carrier and covered by soil. Intranasal exposure of any living organism to *P. fluorescens* is not planned at any stage of the release experiment.

Fluorescent pseudomonads have been reported to colonise mucous membranes of immuno-compromised patients (Niels Høiby, oral communication). In fact, the isolation of *Pseudomonas* species such as *fluorescens* and *putida* from the lungs of cystic fibrosis (CF) patients is not uncommon. However, such colonisations have not been described as contributing to ill health. In the case of *P. aeruginosa*, an opportunistic pathogen of the CF lung, the accumulation of mucus in the respiratory

tract is a direct result of the synthesis of alginates by the bacterium, which significantly contributes to reduced lung function. Similar correlations with *P. fluorescens* have not been established. Furthermore, all human populations including cystic fibrosis patients are exposed to bacteria such as *P. fluorescens* in their lives (e.g., when eating uncooked vegetables). So, the practise of inoculation with *P. fluorescens* would not be expected to create a new biohazard.

As demonstrated by temperature-dependent growth curves of *Pseudomonas fluorescens* strain F113 (D. Dowling, unpublished information, data upon request from the notifier), the growth optimum of the host organism is 30C, and the growth rate at 37C is about ½ of the maximum. This supports that the host organism is not adapted to growth in the human body. In the future, we may have to subdivide the species *P. fluorescens* into two, those that are isolated from plant roots, and those that were isolated from human and animal tissues. *P. fluorescens* F113 has been isolated from a sugarbeet root.

In summary, using present knowledge, the host strain *P. fluorescens* F113

- is not expected to infect healthy individuals
- may potentially be found in the lung of immuno-compromised individuals, however without any known adverse effects
- is not expected to have any toxic effects
- is not expected to have any carcinogenic effects
- is not expected to have any allergenic effects

We conclude that *P. fluorescens* F113 is not pathogenic to humans, animals or plants.

8)

a) The generation time of *Pseudomonas fluorescens* in a natural ecosystem is in the order of hours to days.

b) The generation time of *Pseudomonas fluorescens* in the ecosystem where the release is to take place is expected to be in the order of hours to days.

c) *Pseudomonas fluorescens* has an assexual life cycle

d) The factors which affect the reproduction of *Pseudomonas fluorescens* include nutrient limitation in the soil, physical-chemical factors (pH, water and temperature)

9) Survivability

a) No structures enhancing survival or dormancy are acknowledged

b) Factors which determine the survivability of *Pseudomonas fluorescens* include low pH, predators (protozoa etc.), parasites (virus etc.) and adverse climatic conditions. *Pseudomonas fluorescens* can persist at low levels (10^3 cfu/g) in soils for many years.

10) Dissemination

a) Transport of plant roots and soil by humans, animals or natural forces.

b) Known factors which affect micro-organisms spreading also affect the dissemination of *Pseudomonas fluorescens*

11) Neither the parental or recipient organisms has been notified for release in Denmark.

B1. Information relating to the modification

GMO 1 *P. fluorescens* F113::lacZYrif

The aim of this genetic modification was the construction of a rhizosphere colonising bacterium with a non-antibiotic resistance genetic marker.

A transposable (based on Tn5) vector was used to insert the lacZY genetic marker genes into the host bacterium. The construction of this vector and its insert (pUTlacZY) and its use to construct the GMO is described by Fedi *et al*. (1996) The pUT vectors were developed by Herrero *et al*. 1990; De Lorenzo *et al*. 1990.

These vectors can be used to transfer inserts to the chromosomes of most Gram negative eubacteria by a simple conjugation procedure. The vector component of pUTlacZY does not contribute to any phenotype in the final construct , indeed the only Remaining portions present in GMO are the 19bp inverted repeats from Tn5.

There are no known pathogenicity determinants encoded by any of the vectors used. No vector sequences are present in this GMO.

The plasmid replicon / delivery system and the Tn5 transposase are lost during construction and are not present in the GMO (Herrero *et al*. 1990) see fig 2

The genetic marker or insert is derived as a 6 Kbp DNA fragment from plasmid pGD926 (Ditta *et al.* 1985) containing the *lac ZY* genes from *Escherichia coli K12*. (see Fedi *et al*. 1996) and DNA accession no.PID:g146576.
These genes allow the GMO to utilise lactose as a sole carbon and energy source (see Fig 1&2 and HTML documents below) by virtue of encoding a β-galactosidase and a lactose permease absent in the host strain. The insert is located on the chromosome of the GMO and is expressed constitutively from a native promoter.

Fig 1 Genetic map of *lac* operon

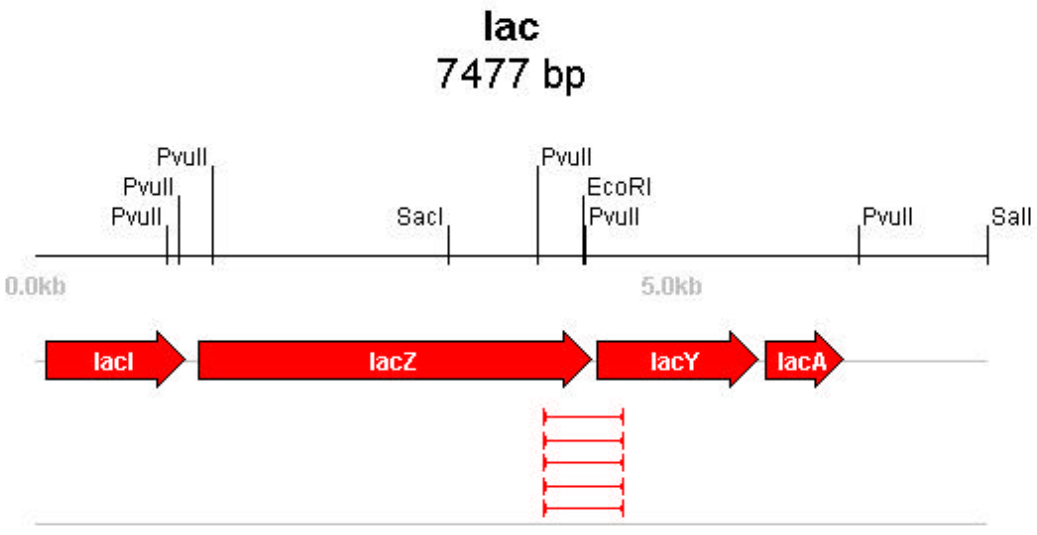
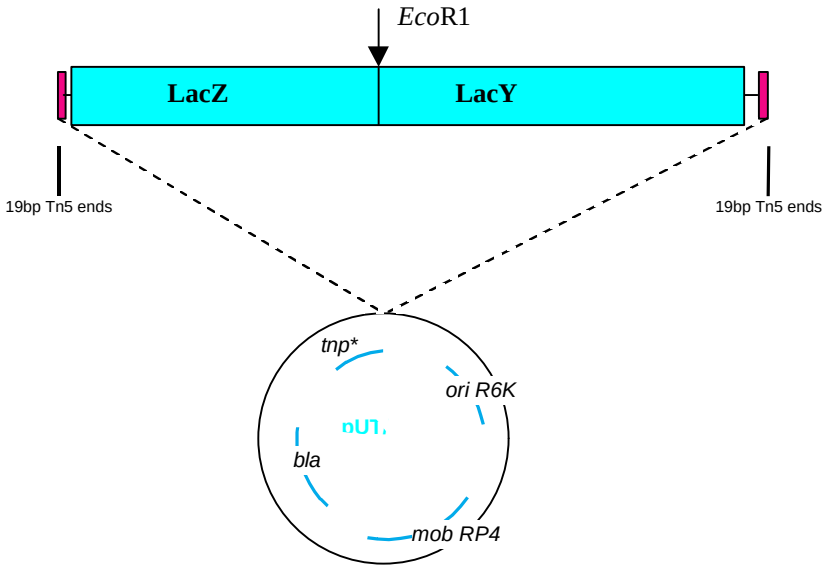


Fig 2 Genetic organisation of *lacZY* insert and delivery vector (from Fedi *et al*. (1996).



pUTLacZY

It is important to note that only the insert (i.e.LacZY) is present in the GMO.

The genetic insert does not code for factors related to human, animal or plant disease, such as

- adherence to cells
- invasion of cells
- cell damage
- sensitivity to antibiotics
- allergens

For further technical details, please refer to Annex B1.

B2. Information relating to the modification

GMO 2 *P. fluorescens* F113rifpcb

The aim of this genetic modification was the construction of a rhizosphere colonising bacterium with the ability to metabolise biphenyl and PCBs

A transposable (based on Tn5) vector was used to insert the *bph* operon from LB400 into the host bacterium. The construction of this vector pUTPtt^{r} (Herrero *et al*. 1990) and its insert (pDDPCB) and its use to construct the GMO is described by Dowling *et al*. 1993 and Brazil *et al*. 1985. The pUT vectors were developed by Herrero *et al*. 1990; De Lorenzo *et al*. 1990.

These vectors can be used to transfer inserts to the chromosomes of most Gram negative eubacteria by a simple conjugation procedure. The vector component of pDDPCB does contribute a Bialaphos (herbicide) resistant phenotype in the final construct. Under natural conditions, most bacteria, including the wildtype strain, can become resistant to Bialaphos spontaneously at a high frequency (10^{-3}-10^{-4}). Bialaphos herbicide resistance in this construct was derived from *Streptomyces hygroscopicus*. (DNA Sequence accession no. XO5822) (Thompson *et al*. 1987).

There are no known pathogenicity determinants encoded by any of the vectors used. Apart from the herbicide resistance gene, no vector sequences are present in this GMO.

The plasmid replicon / delivery system and the Tn5 transposase are lost during construction and are not present in the GMO (Herrero *et al*. 1990) (see Fig. 2).

The *bph* operon or insert consists of a 13.5 Kbp DNA fragment from plasmid pDD530 containing the *bph* operon from *B.cepacia* LB400. (Dowling *et al*. 1993) this insert DNA is derived from 2 sources;

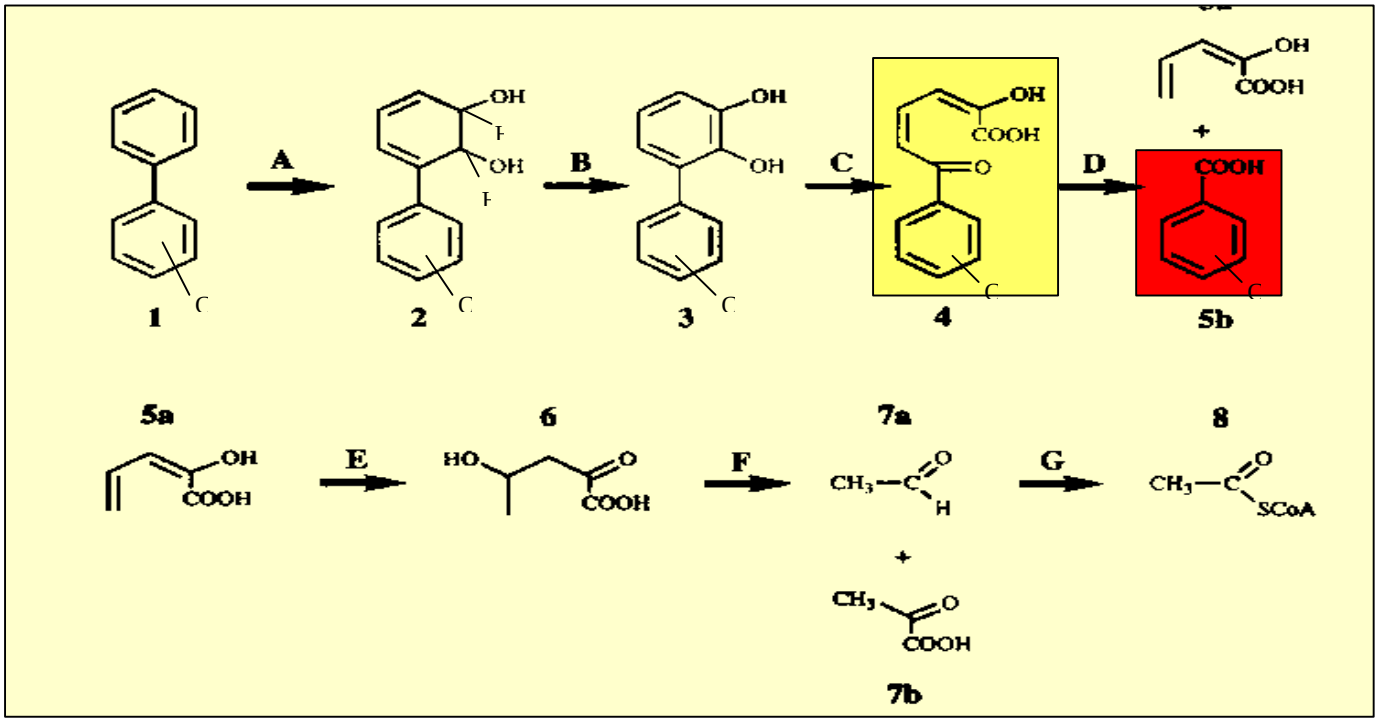
1) 12.5 Kbp fragment from *B.cepacia* LB400
(DNA accession no. X76500, X66122, M86348) Refs. (Erickson and Mondelo, 1992; Dowling *et al*. 1993; Hofer *et al*. 1993; Hofer *et al*. 1994)

2) 1 Kbp fragment from intermediate vector RK2 (Pansegrau *et al*. 1994) (DNA accession no L27758) an artefact of the cloning/recombination process. (No detectable phenotype)

The function of the insert in the GMO allows the host to utilise biphenyl as a sole carbon and energy source and co-metabolise PCBs (see Fig. 1 and Table 1).

The insert is stably located in the chromosome of the host organism and contains 2 regions of unknown function;
1) OrfO - function is unknown but thought to be involved in *bph* operon regulation
2) 1kbp fragment from broad host range plasmid RK2 (an artefact of the intermediate cloning process) has unknown phenotype.

Fig 1. Biochemical pathway for biphenyl/PCB metabolism



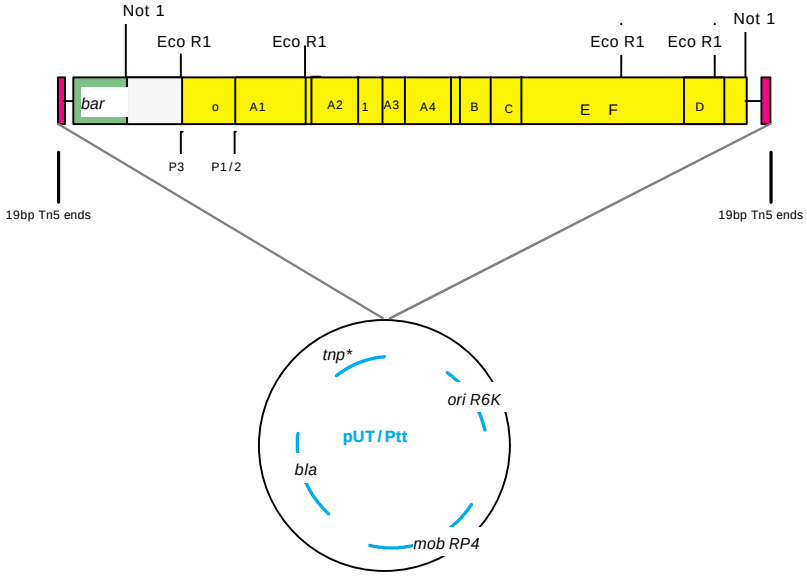
The genes and their products involved in each pathway step are described below in Table 1. Substrates and intermediates are as follows;
1) biphenyl; 2) biphenyl-2,3-dihydro-2,3-diol; 3) biphenyl-2,3-diol; 4) 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid; 5a) 2-hydroxypenta-2,4-dienoic acid' 5b) benzoic acid; 6) 4-hydroxy-2-oxovaleric acid; 7a) acetaldehyde; 7b) pyruvic acid; 8) Acetyl Co-A.

Table 1 Pathway genes, products and function (from Kimura *et al.* 1997)

Pathway step (Fig1)	**Gene**	**Product**	**Length**
	orfO	Putative transcriptional regulator	245aa
A	*bphA*$_1$	Terminal dioxygenase large subunit	459aa
A	*bphA*$_2$	Terminal dioxygenase small subunit	
A	*BphA*$_3$	ferredoxin	109aa
A	*BphA*$_4$	Ferredoxin dioxygenase	408aa
B	*bphB*	Dihydrodiol dehydrogenase	277aa

C	*bphC*	2,3-dihydroxybiphenyl dioxygenase	298aa
D	*bphD*	2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase	286aa
	bphK	Gluthathione S-transferase	203aa
E	*bphH*	2-hydroxy-penta- 2,4-dienoate hydratase	260aa
F	*bphJ*	Aldehyde dehydrogenase	304aa
G	*bphI*	4-hydroxy-2-oxovalerate aldolase	346aa

Fig 2 Genetic organisation of insert and delivery vector (from Dowling *et al.* 1993)



pDDPCB
(20kb)

The *bph* genes are thought to, in terms of bacterial evolution, be derived from other genes, whose gene product has a related environmental function. They are related to genes involved in the breakdown of other aromatics such as toluene, benzene and napthalene. The function of one gene of the *bph* operon, *bph*K may have a "protection" role. Biphenyl is a natural compound found in plants and soil and it is thought that the *bph* genes have evolved to allow bacteria to utilise this substrate. The ability of some *bph* alleles to co-metabolise PCBs is fortunate and due to relaxed substrate specificity of some enzymes in the pathway.

The genetic insert does not code for factors related to human, animal or plant disease, such as

- adherence to cells
- invasion of cells
- cell damage
- sensitivity to antibiotics
- allergens

For further technical details, please refer to Annex B2.

C1. Information on the Organism(s) from which the insert is derived (Donor)

GMO 1 *P. fluorescens* F113::lacZYrif

The insert is ultimately derived from the bacterium *Escherichia coli* K12, a model laboratory strain. This particular laboratory strain is not pathogenic and should be considered a Group 1 organism.

The donor strain species, *E. coli*, is classified under the existing Community rules relating to the protection of human health, in Denmark and at the EU level, as a Group 2 organism, "except for strains not causing disease".

In Germany, the species *E. coli* is classified in risk group 2. (Berufsgenossenschaft der chemischen Industrie. 1997. Sichere Biotechnologie, Eingruppierung biologischer Agenzien: Bakterien. Merkblatt B 006, 2/97, ZH 1/346. Jedermann-Verlag, Heidelberg).

According to the information service of the Office of Biosafety Canada (http://www.hc-sc.gc.ca/hpb/lcdc/biosafty/index.html), the pathogenic strains of *E. coli* are also classified as Group 2 organisms in Canada, by the NIH, by the state of Belgium, and in Australia. However, the NIH classifies strain K12 as a Group 1 organism.

In a Danish review (Christiansen *et al*. 1991. Eksempler på risikoanalyse af værtsmikroorganismer. Miljøprojekt nr. 183, Miljøstyrelsen, København.) it is suggested to classify *Escherichia coli* strain K12 as a Group 1 organism.

Both donor and recipient organism can exchange genetic information under laboratory conditions.

The insert does not contain any factors related to pathogenicity.

The insert does not contain any factors that cause the GMO to have surface properties, including colonising ability of living or dead surfaces, that are different from the wildtype organism.

C2. Information on the Organism(s) from which the insert is derived (Donor)

GMO 2 *P. fluorescens* F113rifpcb

The insert is ultimately derived from the bacterium *Burkholderia* (formerly *Pseudomonas*) *cepacia* (formerly strain) LB400, a common soil microorganism. See (http://www.ncbi.nlm.nih.gov/htbin-post/Taxonomy/wgetorg?id=36873) and the extensive bibliography on strain LB400 in Part 2 of the References section, in particular Bopp (1986).

It is not known if this strain is pathogenic. The genus *Burkholderia* does contain members that are pathogenic to plants and susceptible humans. However the insert (bph operon) does not contain any genes involved in pathogenesis.

The donor strain species, *B. cepacia*, is not classified under the existing Community rules relating to the protection of human health, neither in Denmark nor at the EU level.

In Germany *B. cepacia* is classified in risk group 2. (Berufsgenossenschaft der chemischen Industrie. 1997. Sichere Biotechnologie, Eingruppierung biologischer Agenzien: Bakterien. Merkblatt B 006, 2/97, ZH 1/346. Jedermann-Verlag, Heidelberg).

In other parts of the world, *B. cepacia* has not been considered for inclusion in the list of classified organisms. According to the information service of the Office of Biosafety Canada (http://www.hc-sc.gc.ca/hpb/lcdc/biosafty/index.html), *B. cepacia* is not classified in Canada, by the NIH, by the EU, by the state of Belgium, or in Australia.

The insert does not contain any factors that cause the GMO to have surface properties, including colonising ability of living or dead surfaces, that are different from the wildtype organism.

Both donor and recipient organism can exchange genetic information under laboratory conditions.

D1. Information relating to the genetically modified organism:
GMO 1 *P. fluorescens* F113::lacZYrif

1) Genetic traits and phenotypic characteristics of the GMO:

a) Is the GMO different from the recipient as far as survivability is concerned?
Conclusive results demonstrated by Fedi *et al.* (1996) have shown that the survivability of the GMO *Pseudomonas fluorescens* F113lacZY is the same as the wild type.

b) Is the GMO in any way different from the recipient as far as mode and/or rate of reproduction is concerned?
Conclusive results demonstrated by Fedi *et al.* (1996) have shown that the mode and/or rate of reproduction of the GMO *Pseudomonas fluorescens* F113lacZY is the same as the wild type.

c) Is the GMO in any way different from the recipient as far as dissemination is concerned?
Differences in the dissemination between the wild type and the GMO have not been observed (Sheehan & O'Gara, unpublished).

2) Genetic stability of the GMO:

In numerous lab-based experiments, including specific studies to address stability, the insert has not shown any evidence of genetic instability (Corich *et al.*, 1994; Fedi *et al.*, 1995; Fedi *et al.*, 1996). The genetic insert is located in the chromosome in one copy per genome.

3) Possible pathogenicity of the GMO:

Not known, but unlikely. There is no evidence that the GMO shows plant or animal pathogenicity. Indeed, indigenous fluorescent Pseudomonads are present in high numbers on the roots and surrounding soil of all plants investigated, including vegetable crops.

4) Description of identification and detection methods:

a) Techniques used to detect the GMO in the environment
- Viable plate counting using selective media SA + Rif
- Strain F113lacZYrif can be detected as blue colonies when plated on selective media SA containing rifampicin (50 mg/l) and X-Gal (40 mg/L).
- In addition, a titration plate-based most probable number (MPN) system was developed to recover and estimate the introduced *P. fluorescens* F113 which harbour the lacZY genes from the plant rhizosphere (Fedi *et al.*, 1995).

b) Techniques used to identify the GMO:
- Physiological analysis of key phenotypic properties, e.g., biocontrol activity DAPG and HCN production, and growth on lactose
- Metabolic profiling using BIOLOG
- RAPD analysis using previously evaluated primers and conditions
- rRNA subunit sequence analysis

D2. Information relating to the genetically modified organism:

GMO 2 *P. fluorescens* F113rifpcb

Genetic traits and phenotypic characteristics

Apart from the fact that the GMO can now grow on biphenyl as a sole carbon and energy source and metabolise certain PCBs, the GMO behaves in a similar manner to the original recipient for all parameters examined. Published data indicate that the strain is no more competitive than the wildtype in non-sterile soil microcosms.(Ramos *et al.* 1994; Brazil *et al.* 1995). The mode and rate of reproduction are similar to the parent (recipient). (Ramos *et al.* 1994; Brazil *et al.* 1995). Likewise dissemination of GMO is expected to be no different than recipient. In numerous lab-based experiments including specific studies to address genetic stability of the GMO, the insert has not shown any evidence of genetic instability. (Ramos *et al.* 1994; Brazil *et al.* 1995: D.N.Dowling unpublished observations). The genetic insert is located in the chromosome in one copy per genome.

There is no evidence that the GMO shows plant or animal pathogenicity. Indeed, indigenous fluorescent Pseudomonads are present in high numbers on the roots and surrounding soil of all plants investigated.

The GMO can be detected in the environment by the following methods

1) Viable plate counting using selective media
SA + Rif and colorimetric spray test of viable plate counts
(sensitive to ~10^2 culturable cells/g soil or root)
2) Detection of presence of the *bphC* gene by direct amplification using specific primers from soil extracted DNA.

Techniques used to identify the GMO
1) Physiological analysis of key phenotypic properties
E.g. Biocontrol activity DAPG and HCN production
Growth on biphenyl
2) Metabolic profiling using BIOLOG
3) RAPD analysis using previously evaluated primers and conditions
4) rRNA subunit sequence analysis

D3. Information relating to the two genetically modified organisms:

GMO 1 *P. fluorescens* F113::lacZYrif and **GMO 2 *P. fluorescens* F113rifpcb**

Notifier's assessment of the two GMOs' classification under the existing Community rules relating to the protection of human health:

The recipient organism is classified as Group 1 (see discussion in section A). It does not possess pathogenic or otherwise harmful properties. Episodes of secondary infection in immuno-deficient patients have not given evidence for illness caused by *P. fluorescens*, and none of the bacterial properties of *P. fluorescens* suggests that their might be.

Therefore, the recipient organism can be considered safe with respect to human, animal and plant health.

While the donor species are classified in Group 2 (although Group 1 is appropriate for at least one of the two specific strains used; see sections C1 and C2), the donated materials do not contain pathogenic or otherwise harmful properties (see discussion in sections B1 and B2).

The vectors used do not contain pathogenic or otherwise harmful properties, nor environmentally harmful properties. There are no known pathogenicity determinants encoded by any of the vectors used. Also -- apart from the herbicide resistance gene in the case of F113rifpcb -- no functional vector sequences are present in the GMOs. It is to be noted that no antibiotic resistance determinants were used in the construction of these GMOs.

The GMOs are not expected to cause diseases or illnesses in humans, animals or plants.

Therefore, the two GMOs had been classified by the notifiers as Group 1 organisms.

Re-evaluation at the time of release notification:

The GMOs have been used regularly for several years in several European laboraties under Class 1 conditions, without any evidence of adverse effects on humans or plants. No new evidence suggests that the classification should be changed.

E. Information relating to the release

1) The general purpose of the release is to document the safety of GMO bioremediation strains based on *Pseudomonas fluorescens* strain F113. The technological perspective is the development of genetically modified microbial technology addressing the problem of contaminated soil.

The specific purpose of the release is to answer the following research questions:

- Will the bacterial delivery system function physiologically (compatibility of plant plus bacterium in contaminated soil), ie., will the genetically modified bacterial inoculum colonise the plant root and survive in contaminated soil under field conditions?
- Will the biological system function physiologically, ie, will *bph* genes give a survival advantage in contaminated soil under field conditions?
- Does the genetically modified system perform better than existing non-genetically modified inocula under field conditions (comparison of LB400's and F113pcb's functional traits for survival and bioremediation)?

2) The site of the release is different from the natural habitat where the recipient organism is regularly used or found. The natural habitat of strain F113 is (non-contaminated) agricultural soil, in particular plant root surfaces within such soil. The difference at the release site is the contamination of the soil with PCBs. The effect of the contamination on the genetically modified strains' survival is the research topic of the release.
Soil analyses, performed in 1999, identified the top 50-cm layer of the release site to be contaminated with 1.4 mg/kg total PCBs (analysis report in Appendix E).

3) Release site and surrounding area:

a) The geographical location of the release is the topsoil of a corner (Danish GIS gridline reference 565995; 6217373) of a scrapmetal recycling yard (Fig. 1) owned by Uniscrap A/S Genvindingsindustri, Kathale 36, DK-6200 Aabenraa.
The release site's address is:

Uniscap A/S
Beringvej 45
DK-8464 Hasselager

The administrative region is Aarhus Amt, Denmark.

b) The size of the release site is 81 m^2. The wider release area is 4000 m^2, which is the size of the part of the scrapmetal yard that has been planted with willow in the spring of 1999 (Fig. 1).

c) There are no internationally recognized biotopes or protected areas in the proximity. The distances, as the crow flies, to the nearest internationally recognized biotopes (as proposed by Denmark on its list of 194 "EF-habitatområder") are 14 km (Mossø, WSW), 25 km (Tved Kær, ENE), 27 km (Sydlige Helgenæs, E), and 28 km (Silkeborg Skove, W).

Information on other protected areas (”Paragraf 3 områder”) , including water reservoirs, within 6 km of the release site is presented in Appendix E (distances between release site and these protected are presented in the form of two maps and a table).

d) The surrounding area consists to the East and North of commercial buildings, driveways and barren soil used for scrap storage (contaminated with up to 7600 mg/kg total hydrocarbons and 1100 mg/kg total cyanide, potentially contaminated with PCBs; analysis report in Appendix E), and to the South and West of uncultivated grass, bush and single tree vegetation (no history of contamination).

The release site can be regarded as a contained site in relation to surface migrating animals, as it will be surrounded by a fence.

4) Method and amount of release:

a) Number of genetically modified bacteria to be released:

Plants to be used:
Willow and alfalfa. All inoculation will be performed on cuttings or seeds.

Willow cuttings: 10^{10} cells / willow plant, 2 plants / m^2.
450 kg soil / m^2 at 30 cm top layer thickness = $0.5*10^5$ cells / g soil.
30 m^2 with willow = 60 plants = $6*10^{11}$ cells

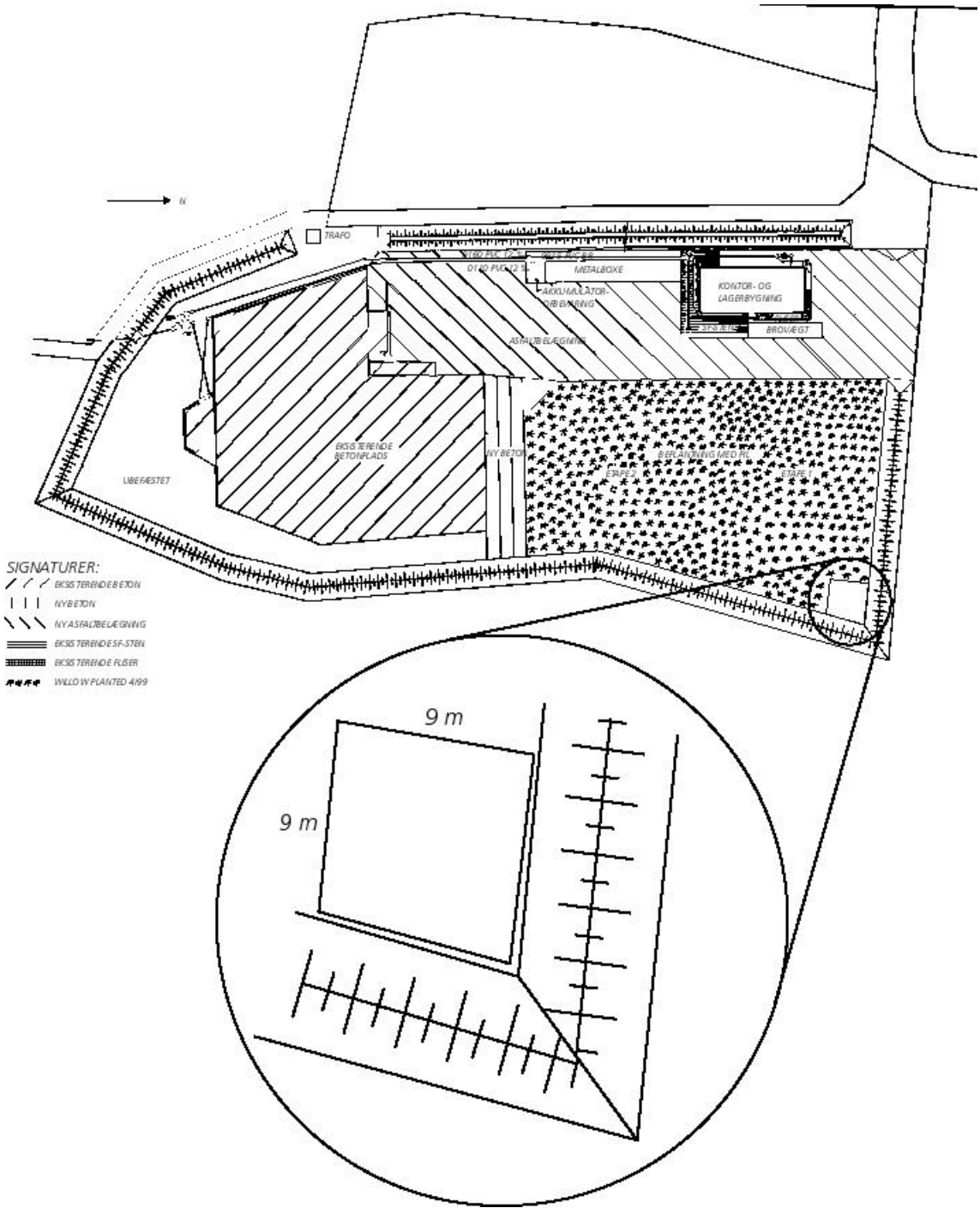
Alfalfa seeds: 10^6 cells / alfalfa seed, 1000 seeds / m^2.
450 kg soil / m^2 at 30 cm top layer thickness = $2.5*10^3$ cells / g soil.
30 m^2 with alfalfa = 30000 seeds = $0.3*10^{11}$ cells.

Total: $6.3*10^{11}$ genetically modified bacterial cells to be released, half of them strain F113::lacZYrif, and the other half strain F113rifpcb.

b) The release is to be performed within one day during daylight.
In preparation of the release, plants and seeds will be inoculated with the appropriate GMO bacteria at the laboratory of NERI, Roskilde. On the day of the release, inoculated materials will be packaged in suitable materials and transported to the release site by car.

c) To minimize spread of the GMOs, the genetically modified bacterial cells will be immobilised in a seed pelleting carrier and coated around the cuttings or seeds prior to the release (this procedure is to be performed in a closed GMO laboratory at NERI, Roskilde). The release operation will involve removing the coated seeds or cuttings from packages, sticking them into the soil and covering with soil. Contact of bacteria with the air will be minimum. Bacteria will not intentionally be spread on the soil surface. No aerosols will be formed at the site.

Fig. 1: Map of the Hasselager, Århus, release trial site. The release site measures 9 x 9 m.



F. Interactions of the GMOs with the environment and potential impact on the environment.

The genetically modified microorganisms proposed to be released are derivatives of *Pseudomonas fluorescens* F113 and should behave identically to the non-modified parental strain in relation to its interactions with other organisms. Strain F113 was isolated from the sugar beet rhizosphere because of its potential use as a biocontrol agent and can interact with other organisms because of its ability to colonize the plant rhizosphere.

These traits have been extensively studied *in vitro*, in microcosm models and in a previous deliberate release (O'Gara *et al.* 1994; Dowling *et al.* 1995; Corich *et al.* 1995).

Experiments have demonstrated that F113 can colonize the rhizosphere of virtually any plant: published research has shown colonization of the sugarbeet (Carroll *et al.* 1995), pea (Naseby and Lynch, 1999) and tomato (Chin-A-Woeng *et al.*, 1997) rhizosphere. Unpublished results show equivalent colonization of the alfalfa (Dowling and Rivilla, unpublished, Appendix D) and willow (Appendix D) rhizosphere.

The F113 production of HCN can affect plant respiration. Experiments performed in gnotobiotic systems with alfalfa (no soil, only 2 bacterial strains, *Rhizobium* sp. and strain F113) have shown that plants inoculated with F113 are significantly smaller than non inoculated plants (Rivilla unpublished). However in soil microcosms similar experiments do not show this deletereous effect (Dowling, unpublished). It is likely that soil autochthonous microorganisms compete with F113 limiting its population and therefore its effects in the plant. For this reason it is highly unlikely that in a deliberate release in natural soil, F113 derivatives should negatively affect plants.

Experiments have been also performed to test the effect of F113 and derivatives on plant microsymbionts that indirectly may affect plant growth. F113 did not show any effect on the *Sinorhizobium meliloti*-alfalfa symbiosis (Rivilla, unpublished). Nodule numbers, plant nitrogen contents and rhizobial root colonization were unaffected in co-inoculated plants. Similarly, no adverse effects were observed on the micorrhizal symbiosis *Glomus mosseae*-tomato when inoculated with F113 and derivatives, including a DAPG overproducer strain (Barea *et al.* 1998).

F113 derivatives might adversely affect soil microorganisms. As reported above no such effects have been observed on beneficial microsymbionts. Furthermore, yeast and fungal colony forming units were not significantly affected by F113 and derivatives in a Pea rhizosphere microcosm at any soil pH studied (Naseby and Lynch, 1999). Similarly, no effect has been detected in bacterial colony forming units in the alfalfa rhizosphere (Dowling, unpublished). These results contrast with the biocontrol effect that F113 exerts on plant pathogenic fungi (Carroll *et al.*, 1995; Dunne *et al.*, 1995) and bacteria (Cronin *et al.*, 1997).

Other possible interaction of F113 derivatives with soil microorganisms is lateral DNA transfer. Insertion of *LacZY* and *bph* genes into F113 has been performed by transposition, generating stable insertions that do not contain the transposase gene (Fedi

et al., 1996; Brazil *et al.*, 1996). However lateral *in vitro* transfer of the *LacZY* genes has been shown at a 10^{-6}- 10^{-7} frequency, while experiments in microcosms showed no transfer (Fedi *et al.*, 1996), indicating that DNA transfer in a natural soil does not occur, or occurs at a very low frequency.

The reports cited above indicate that although F113 might affect some species of microorganisms it would not have an effect on the overall soil microflora and it would not affect key species important because of its beneficial effects in soil. The experience generated by previous deliberate release together with laboratory experiments suggest that interactions of the genetically modified derivative of strain F113 with other organisms are not different from the wildtype strain F113.

Special considerations for the contaminated soil environment at the release site:
Strain F113pcb produces chlorobenzoates (CBs) from PCBs. The CBs are terminal metabolites in this genetically modified strain. As F113pcb will predominantly grow on the root surfaces of experimental plants, the primary ecological interaction of the CBs is expected to be with these plants at the contaminated release site, and with the contaminated soil around the plant roots at the release site. Metabolites cannot be produced outside the release area, as those soils are not contaminated, i.e., do not provide a substrate for the production of CBs, which excludes unintended effects outside the release area. Recent results (Lagergren 1999) suggest that plants readily take up CBs, tolerating surprisingly high concentrations. As CBs enter the plants' metabolism, they act as herbicides at high concentrations. Based on the relatively low PCB concentrations at the release site, rootzone CB concentrations can be expected to be several orders of magnitude lower than in the study cited, i.e., no deleterious effects on plant growth are to be expected. However, it is reasonable to expect that the CBs produced will be removed from the soil environment through plant uptake.

Conclusion: Unintended ecosystem effects are not to be expected from a bacterium modified to degrade the soil pollutant PCBs. At the contaminated site the metabolites formed will not be released to non-contaminated soil layers or groundwater.

Suggested monitoring: Soil porewater analyses at and below rootzones (at and below PCB-contaminated soil layers) in order to confirm the absence of water-soluble CBs.

Regarding strain F113rifpcb:

This GMO has not been released in the past. However, published data show that the strain displayed the same kinetics of colonisation of roots and the numbers of bacteria colonised the roots as the wildtype. In addition, it was observed that the colonisation competence in nonsterile soil microcosms of the GMO strain and the wild type are equally competitive in the rhizosphere of sugarbeet (Brazil *et al.* 1995).

Regarding strain F113::lacZYrif

Further details on experiences made during 2 deliberate releases of strain F113lacZY are cited in Appendix F. The only difference between the previously released strain, and the strain F113::lacZYrif of this application, is a Rifampicin resistance achieved by spontaneous mutation.

G. Information relating to monitoring

1) Method for monitoring the GMOs

Soil, root and leaf samples will be taken periodically and analysed. Viable bacterial cells will be counted by selective plating on agar media.

- Media formulation (SA medium):

Sucrose (Saccharose)	20 g/L
Asparagine	2 g/L
Rifampicin	50 mg/L
X-Gal	40 mg/L

- Alternative media formulation:

Lactose as a sole source of organic C, for F113:lacZYrif. The expression of LacY (lactose permease) effects a phenotype that is capable of growing (selectively) on lactose plates.

- Spray test:

2,3-dihydroxy-biphenyl	0.1% wt/vol., spray with atomiser

Detection and quantification: Low iron SA rif Xgal plates will be used to distinguish between natural soil microflora and the two released strains:

a. non-fluorescent colonies + spray test positive = rifampicin resistant PCB-degraders, e.g. *Burkholderia cepacia* LB400

b. fluorescent colonies + spray test positive = *P. fluorescens* F113rifpcb

c. blue colonies = *P. fluorescens* F113::lacZYrif

Alternatively, an MPN method (Fedi *et al*. 1996) targeting the phenotypic expression of the lacZY genes can be employed to quantify strain F113::lacZY.

Additional PCR-based detection system (lacZY & EcoR1 restriction):
Soil, root and leaf sampling will be performed at the release site and its surroundings. Total DNA will be extracted. DNA samples will be processed by PCR, targetting specific regions of the donated genes. Specific DNA probes will be used to visualize PCR products derived from the donated genes.

2) Methods for monitoring ecosystem effects

Unintended ecosystem effects are not to be expected outside the release area. Potential effects are limited to the presence of the soil contaminant type, PCBs. Soil porewater analyses at and below PCB-contaminated soil layers (at 20, 50 and 100 cm depth) will be performed confirming the absence of water-soluble CBs.

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

Organisms considered: Other bacteria.
Soil, root and leaf sampling will be performed at the release site and its surroundings. From these samples, bacterial colonies will be grown on nutrient media. Colonies will be blotted and lysed. Specific DNA probes will be used to visualize DNA derived from the donated genes. Donated DNA detected in colonies that do not fulfil the requirements under 1a.-1c. will be considered recipient organisms of tranfered DNA.

4) Spatial extent of the monitoring area

500 m in all directions

5) Duration of the monitoring

During the experiment (1 to 2 seasons) plus 5 years thereafter. To be extended according to environmental needs.

6) Frequency of the monitoring

1 x per month, during the first 6 months.
1 x every 3 months during the period month 7—18 post release.
2 x per year thereafter.

H. Information on post-release and waste treatment

1) Post-release treatment of the site

None.

2) Post-release treatment of the GMOs

None.

3) Waste: type, amount and treatment

Waste: Packaging of materials used on the day of the release; a few kg.
Samples taken during monitoring, 10—100 kg per sampling, see part G.6 for monitoring schedule.

Treatment: All waste will be collected in plastic bags and transported by car to the laboratory at NERI, Roskilde, which is approved for work with GMOs. Ultimately, everything will be destroyed by autoclaving.

I. Information on emergency response plans

1) Methods and procedures for controlling GMOs in case of unexpected spread

Spraying of vegetation with Roundup, thereby eliminationg the host plants for the derivatives of strain F113. In addition, strain F113 is Roundup-sensitive (D. Dowling, unpublished), i.e., survival of F113 derivatives will be reduced.

2) Methods for decontamination of the areas affected

Removal of soil and plants followed by incineration: Plant tops will be harvested, plant main roots with surrounding soil (hot spots of bacterial inoculation) will be dug out, everything transfered into plastic sacks and treated as hospital waste.

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

Autoclaving, incineration.

4) Plans for protecting human health and the environment in case of the occurence of an undesirable effect

See above 1) – 3).

REFERENCES, PART 1:

General Bibliography

(Bibliography on strain LB400 is presented separately, 3 pages down).

Barea, J.M., Andrade, G., Bianciotto,V., Dowling, D., Smith, L.M., O'Gara, F. and Azcon-Aguilar, C. (1998) Impact on arbuscular mycorrhiza formation of *Pseudomonas* strains used as inoculants for biocontrol of soil-borne fungal pathogens. Appl.Environ.Microbiol. 64: 2304-2307.

Bergey, D.H. (1982). Gram-negative aerobic rods and cocci, p. 140-219. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams and Wilkins Co., Baltimore, U. S. A.

Brazil, D., Kenefick,L., Callanan,M., Hara,A., de Lorenzo,V., Dowling,D.N. and O'Gara,F. (1995) Construction of a Rhizosphere Pseudomonad with potential to Degrade PCBs: Detection of bph Gene Expression in the Rhizosphere. Appl. Environ. Microbiol. 61:1946-1952

Carroll, H., Moënne-Loccoz, Y., Dowling, D.N. and O'Gara, F. (1995). Mutational disruption of the biosynthesis genes coding for the antifungal metabolite 2,4-diacetylphloroglucinol does not influence the ecological fitness of *Pseudomonas fluorescens* F113 in the rhizosphere of sugarbeets. Appl. Environ. Microbiol. 61(8): 3002-3007.

Chin-A-Woeng, T.F.C., de Priester,W., van der Bij,A.J. and Lugtenberg.B.J.J. (1977). Description of the colonization of a gnotobiotic tomato rhizosphere by *Pseudomonas fluorescens* biocontrol strain WCS365, using scanning electron microscopy. Mol. Plant-Microbe Interact. 10(1): 79-86.

Corich, V., Giacomini, G., Concheri, G., Ritzerfeld, B., Vendramin, E., Struffi, P., Basaglia, M., Squartini, A., Casella, S. and Nuti, M. P. (1994). Environmental impact of genetically modified *Azospirillum brasilense*, *Pseudomonas fluorescens*, and *Rhizobium legunimosarum* released as soil/seed inoculants. In Proceedings of the 3rd International Symposium on The Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms. Jones D. D. (Ed.) The University of California, Division of Agriculture and Natural Resources Oakland, Calif., U. S. A.

Corich, V., Giacomini, A., Concheri, B. Ritzerfeld, E., Vendramin, E., Struffi, P., Basaglia, M., Squartini, A., Casella, S., Nuti, M.P., Peruch, U., Poggiolini, S., de Troch, P., Vanderleyden, J., Fedi, S., Fenton, A., Moenne-Loccoz, Y., Dowling, D.N. and O'Gara, F. (1995) Environmental Impact of Genetically modified *Azospirillum brasilense, Pseudomonas fluorescens* and *Rhizobium leguminosarum* released as soil/seed inoculants. In: Monterey Biosafety Symposium (ed Jones) University of California, Berkeley.

Cronin, D., Moenne-Loccoz,Y., Fenton,A., Dunne,C., Dowling,D.N., and O'Gara,F. (1997) Ecological interaction of a biocontrol *Pseudomonas fluorescens* strain producing 2,4-diacetylphloroglucinol with the soft rot potato pathogen Erwinia carotovora subsp. atroseptica. FEMS Microbial Ecology 23: 95-106.

De Lorenzo, V., Herrero, M., Jacubzik, U. and Timmis, K.N. (1990) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing and chromosomal insertion of cloned DNA in Gram negative bacteria. J. Bacteriol. 172:6568-6572.

Ditta, GT, Schmidhauser, T., Yalobson, E., Lu,P., Liang,X., Finaly,DR., Guiney,D., and Helinsky,D. (1985). Plasmids related to the broad host range vector pRK290 useful for gene cloning and for monitoring gene expression. Plasmid 13:149-153.

Dowling, D.N., Pipke, R. and Dwyer, D.F. (1993) A DNA module encoding bph genes for the degradation of polychlorinated biphenyls (PCBs). FEMS Micobiology Letters 113:149-154.

Dowling, D.N., Boesten,B., O'Sullivan,D.J., Stephens,P., Morris,J. and O'Gara,F. (1995) Genetically-engineered fluorescent pseudomonads for improved biocontrol of plant pathogens. In: Biocontrol Agents: Benefits and Risks (Hokkanen,H.M.T. and J.M.Lynch, eds.) Ch.14 p161-166. Cambridge University Press.

Dunne, C., Moenne-Loccoz,Y., McCarthy,J., Higgins,J., Powell,J., Dowling,D.N. and O'Gara,F. (1998) Combining proteolytic and phloroglucinol-producing bacteria for improved biocontrol of Pythium-mediated damping-off of sugar beet. Plant Pathology 47: 299-307.

Erickson, B.D., and Mondello,F. (1992) Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in *Pseudomonas* Strain LB400. J.Bacteriol. 174: 2903-2912.

Fedi, S., Moënne-Loccoz, T., Dowling, D. N. and O'Gara, F. (1995). A versatile most probable number system to quantify lacZY-marked pseudomonads present at low cell numbers in the rhizosphere. Letts. Appl. Microbiol. 20, 220-224.

Fedi, S., Brazil, D., Dowling, D.N and O,Gara F. (1996) Construction of a modified mini-Tn5 lacZY non-antibiotic marker cassette: ecological evaluation of a lacZY marked *Pseudomonas* strain in the sugarbeet rhizosphere. FEMS Microbiology Letters 135:251-257.

Herrero, M., deLorenzo, V. and Timmis, K. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram negative bacteria. J.Bacteriol. 172, 5557-6567.

Hofer, B., Backhaus,S. and Timmis,K.N. (1994) The biphenyl/polychlorinated biphenyl-degradation locus (bph) of Pseudomonas sp.LB400 encodes four additional metabolic enzymes. Gene 144: 9-16.

Hofer, B., Eltis,L., Dowling,D.N., and Timmis, K.N. (1993) Genetic analysis of a gene cluster encoding a pathway for biphenyl/polychlorinatedbiphenyl degradation. Gene 130:47-55.

Kimura, N., Nishi, A., Goto, M. and Furukawa, K. (1997) Functional analysis of a variety of chimeric dioxygenases constructed from two biphenyl dioxygenases that are similar structurally but different functionally. J. Bacteriol. 179: 3936-3943.

Lagergren, A. Uptake of chlorinated benzoic acids by *Salix sp.* (willow): Do willow roots remove intermediate products of PCB-metabolism from nutrient solution? M.S. thesis, accepted 12 Nov. 1999, University of Kalmar, Sweden.

Mondello, F. (1989) Cloning and expression in *Escherichia coli* of *Pseudomonas* strain LB400 genes encoding Polychlorinated biphenyl degradation. J. Bacteriol. 171: 1725-1732.

Naseby, D.C. and Lynch, J.M. (1999). Effects of *Pseudomonas fluorescens* F113 on ecological functions in the pea rhizosphere are dependent on pH. Microb. Ecol. 37(4): 248-256.

O'Gara, F., Dowling, D.N. and Boesten, B. (eds.) (1994) Molecular Ecology of Rhizosphere Microorganisms. pp177 , VCH Weinheim Germany.

Pansegrau,W., Lanka,E., Barth,P.T., Figurski,D.H., Guiney,D.G., Haas,D., Helinski,D.R., Schwab,H., Stanisich,V.A. and Thomas,C.M. (1994) Complete nucleotide sequence of Birmingham IncP-alpha plasmids: compilation and comparative analysis J. Mol. Biol. 239, 623-663.

Ramos, J.L., Diaz,E., Dowling,D.,De Lorenzo,V., Molin,S., O'Gara,F., ramos,C., and Timmis,K.N. (1994) The behaviour of bacteria designed for biodegradation. (Nature-) Biotechnology 12: 1349-1356.

Shanahan, P., O'Sullivan, D. J., Simpson, P., Glennon, G. and O'Gara, F. (1992). Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. Appl. Environ. Microbiol. 58, 353-358.

Thompson,C.J., Movva,N.R., Tizard,R., Crameri,R., Davies,J.E., Lauwereys,M. and Botterman,J. (1987) Characterization of the herbicide-resistance gene bar from *Streptomyces hygroscopicus* EMBO J. 6, 2519-2523 . C.J., Movva,N.R., Tizard,R., Crameri,R., Davies,J.E., Lauwereys,M. and Botterman,J. (1987) Characterization of the herbicide-resistance gene bar from *Streptomyces hygroscopicus* EMBO J. 6, 2519-2523 .

REFERENCES, PART 2:
Bibliography - *Burkholdaria (Pseudomonas) cepacia* LB400

Arnett, C.M., Parales, J.V., Haddock, J.D., (1998), Specificity of the biphenyl-2,3-dioxygenase of Pseudomonas sp. strain LB400 for chlorinated biphenyls, Abstr.Gen.Meet.Am.Soc.Microbiol, 98 Meet., 426-27.

Aronstein, B.N., Rice, L.E., (1996), Biological and integrated chemical-biological treatment of PCB congeners in soil sediment containing systems, International Journal of Multiphase flow, 22; Supplement 125,

Barriault, D., Pelletier, C., Hurtubise, Y., Sylvestre, M., (1997), Substrate Selectivity Pattern of Comamonas testosteroni Strain B-356 towards Dichlorobiphenyls, International Biodeterioration & Biodegradation, 39; 311-16.

Bedard, D.L., Advan. Appl. Biotechnol. Ser., (1990), Bacterial transformation of polychlorinated biphenyls, Biotechnol.Biodegradation, 369-88

Bergeron, J., Ahmad, D., Barriault, D., Larose, A., Sylvestre, M., Powlowski, J., (1994), Identification and mapping of the gene translation products involved in the first steps of the Comamonas testosteroni B-356 biphenyl/chlorobiphenyl biodegradation pathway, Can.J.Microbiol, 40; 743-53

Bergeron, J.A., Bopp, L.H., (1991), Kinetics of PCB uptake and degradation, Abstr. Gen. Meet. Am. Soc. Microbiol., 91 Meet.; 285.

Billingsley, K. A., Backus, S.M., Juneson, C., Ward, O.P., (1997), Comparison of the degradation patterns of polychlorinated biphenyl congeners in Aroclors by Pseudomonas strain LB400 after growth on various carbon sources, Canadian Journal of Microbiology, 43;1172-1179.

Billingsley, K.A., Backus, S.M., Juneson, C., Ward, O.P., (1997), Comparison of the degradation patterns of polychlorinated biphenyl congeners in Aroclors by Pseudomonas strain LB400 after growth on various carbon sources, Can.J.Microbiol, 43; 1172-79.

Billingsley, K.A., Backus, S.M., Ward, O.P., (1997), Studies on the transformation of selected polychlorinated biphenyl congeners by Pseudomonas strain LB400, Can .J. Microbiol, 43; 782-88

Blasco, R., Mallavarapu, M., Wittich, R.M., Timmis, K.N., Pieper, D.H., (1997), Evidence that formation of protoanemonin from metabolites of 4-chlorobiphenyl degradation negatively affects the survival of 4-chlorobiphenyl-cometabolizing microorganisms, Appl. Environ. Microbiol, 63; 427-34.
Bopp, L.H., (1985), Regulation of polychlorinated biphenyl degradation in Pseudomonas strain LB400, Abstr. Annu. Meet. Am. Soc. Microbiol., 85 Meet.; 266

Bopp, L.H., (1986), Degradation of highly chlorinated PCBs by Pseudomonas strain LB400, J. Ind. Microbiol., 1; 23-29.

Bopp, L.H., Bedard, D.L., Haberl, M.L., (1986), Bacterial degradation of individual

polychlorinated biphenyl congeners in an assay mixture is influenced by the other congeners present, Abstr. Annu. Meet. Am. Soc. Microbiol., 86 Meet.; 294

Bruehlmann, F., Chen, W., (1999), Tuning biphenyl-dioxygenase for extended substrate specificity, Biotechnol.Bioeng, 63; 544-51.

Chung, S.Y., Maeda, M., Song, E., Horikoshi, K., Kudo, T., (1994), A Gram-positive polychlorinated biphenyl-degrading bacterium, Rhodococcus erythropolis strain TA421, isolated from a termite ecosystem, Biosci.Biotechnol.Biochem., 58; 2111-13.

De Lorenzo, V., Eltis, L., Kessler, B., Timmins, K.N., (1993), Analysis of Pseudomonas gene products using lacIq/Ptrp-lac plasmids and transposons that confer conditional phenotypes, Gene, 123; 17-24.

Donnelly, P.K., Hegde, R.S., Fletcher, J.S. (1994), Growth of PCB-degrading bacteria on compounds from photosynthetic plants, Chemosphere, 28; 981-88.

Dowling, D.N., Pipke, R., Dwyer, D.F., (1993), A DNA module encoding bph genes for the degradation of polychlorinated biphenyls (PCBs), FEMS-Microbiol.Lett.,113; 149-54.

Eltis, L.D., Bolin, J.T.,(1996), Evolutionary relationships among extradiol dioxygenases, Journal of Bacteriology, 178; 5930

Eltis, L.D., Hofmann, B., Hecht, H.J., Lunsdorf, H., Timmis, K.N., (1993), Purification and crystallization of 2,3-dihydroxybiphenyl 1,2- dioxygenase, J. Biol. Chem, 268: 2727-2732.

Erb, R.W., Wagner-Dobler, I., Detection of polychlorinated biphenyl degradation genes in polluted sediments by direct DNA extraction and polymerase chain reaction, Appl. Envir. Microbiol. 59: 4065-4073.

Erickson, B.D., Mondello, F.J., (1992), Nucleotoide Sequencing and Transcriptional Mapping of the Genes Encoding Biphenyl Dioxygenase, a Multicomponent Polychlorinated-Biphenyl-Degrading Enzyme in Pseudomonas Strain LB400, Journal of Bacteriology, 174; 2903-12.

Erickson, B.D., Mondello, F.J., (1993), Enhanced biodegradation of polychlorinated biphenyls after site- directed mutagenesis of a biphenyl dioxygenase gene, Appl. Envir. Microbiol. 59: 3858-3862.

Evans, B.S., Dudley, C.A., Klasson, K.T., (1996), Sequential anaerobic-aerobic biodegradation of PCBs in soil slurry microcosms, Appl Biochem Biotechnol, 57-58; 885-94.

Favaloro, B., Tamburro, A., Angelucci, S., Luca, A.D., Melino, S., di Ilio, C., Rotilio, D., (1998), Molecular cloning, expression and site-directed mutagenesis of glutathione S-transferase from Ochrobactrum anthropi, Biochem J., 335; 573-9

Fukuda, M., Yasukochi, Y., Kikuchi, Y., Nagata, Y., Kimbara, K., Horiuchi, H., (1994), Identification of the bphA and bphB genes of Pseudomonas sp. strain

KKS102 involved in degradation of biphenyl and polychlorinated biphenyls, Biochem. Biophys. Res. Commun, 202; 850-56.

Furukawa, K., (1995), Construction and functional analyses of hybrid aromatic ring dioxygenases, J. Cell. Biochem, Suppl.21A; 38

Gibson, D.T., Cruden, D.L., Haddock, J.D., Zylstra, G.J., Brand, J.M., (1993), Oxidation of polychlorinated biphenyls by Pseudomonas sp. strain LB400 and Pseudomonas pseudoalcaligenes KF707, J Bacteriol, 175; 4561-4

Gibson, D.T., Cruden, D.L., Haddock, J.T., Zylstra, G.J., Brand, J.M., (1993), Oxidation of polychlorinated biphenyl by Pseudomonas sp. Strain LB400 and Pseudomonas pseudoalcaligenes KF707, Journal of Bacteriology, 175; 395-410

Gibson, D.T., Torok, D.S., Lee, K., Resnick, S.M., Haddock, J.D., (1995), Multiple reactions catalyzed by multicomponent aromatic hydrocarbon dioxygenases, Cell. Biochem, Suppl.21A; 40.

Habe, H., Kimura, T., Nojiri, H., Yamane, H., Omori, T., (1996), Cloning and nucleotide sequences of the genes involved in the meta-cleavage pathway of cumene degradation in Pseudomonas fluorescens IP01, J. Ferment. Bioeng, 81; 247-54

Haddock, J. D., Gibson, D. T., (1995), Dihydroxylation and dechlorination of chlorinated biphenyls of purified biphenyl 2,3 dioxygenase from Pseudomonas sp. strain LB400, Journal of Bacteriology, 177; 20

Haddock, J. D., Gibson, D. T., (1995), Purification and characterization of the oxygenase component of biphenyl 2,3-dioxygenase from Pseudomonas sp. strain LB400, Journal of Bacteriology, 177; 5834

Haddock, J. D., Gibson, D. T., (1996), Purification and characterization of the oxygenase component of biphenyl 2,3-dioxygenase from Pseudomonas sp. strain LB400, Journal of Bacteriology, 178; 2158

Haddock, J. D., Pelletier, D. A., Gibson, D. T., (1997), Purification and properties of ferredoxinBPH, a component of biphenyl 2,3-dioxygenase of Pseudomonas sp.strain LB400, J Ind Microbiol Biotechnol, 19; 355-9.

Haddock, J.D., Nadim, L.M., Gibson, D.T., (1993), Oxidation of biphenyl by a multicomponent enzyme system from Pseudomonas sp strain LB400, Journal of Bacteriology, VOL; PAGE

Haddock, J.D., Nadim, L.M., Gibson, D.T., (1993), Oxidation of biphenyl by a multicomponent enzyme system from Pseudomonas sp. strain LB400, J. Bacteriol, 175; 395-400.

Hernandez, B.S., Koh, S.C., Chial, M., Focht, D.D., (1997), Terpene-utilizing isolates and their relevance to enhanced biotransformation of polychlorinated biphenyls in soil, Biodegradation, 8; 153-58.

Higson, F.K., (1992), Microbial degradation of biphenyl and its derivatives, Adv.

Appl. Microbiol.,37; 135-64.

Hofer, B., Backhaus, S., Timmis, K.N., (1994), The biphenyl / polychlorinated biphenyl degradation locus (bph) of Pseudomonas sp. LB400 encodes four additional metabolic enzymes, Gene, 144; 9-16.

Hofer, B., Eltis, L.D., Dowling, D.N., Timmis, K.N., (1993), Genetic analysis of a Pseudomonas locus encoding a pathway for biphenyl/polychlorinated biphenyl degradation, Gene, 130; 47-55.

Hofer, B., Eltis, L.D., Dowling, D.N., Timmis, K.N., (1992), Genetic analysis of a locus encoding a pathway for biphenyl/polychlorobiphenyl degradation, Biol. Chem. Hoppe-Seyler, 373; 861

Hurtubise, Y., Barriault, D., Sylvestre, M., (1998),Involvement of the terminal oxygenase beta subunit in the biphenyl dioxygenase reactivity pattern toward chlorobiphenyls, Journal of Bacteriology, 180; 5828.

Hwang, S., Kim, C.K., Kim, Y., Kim, S.J., Kim, Y.C., (1999), The bphIJ genes encoding acetaldehyde dehydrogenase (acylating) and 4-hydroxy-2-oxovalerate aldolase in Pseudomonas sp. DJ77 and their evolutionary implications, Biochem Biophys Res Commun, 256; 469-73.

Khan, A., Nawaz, M., Cerniglia, C., (1994), Purification and characterization of 2,3-dihydroxybiphenyl-1,2-dioxygenase from a polychlorinated biphenyl degrading Pseudomonas putida OU83 strain, Abstr.Gen.Meet.Am.Soc.Microbiol., 94 Meet.; 443.

Khan, A.A., Wang, R., Nawaz, M.S., Cerniglia, C.E., (1997), Nucleotide sequence of the gene encoding cis-biphenyl dihydrodiol dehydrogenase (bphB) and the expression of an active recombinant His-tagged bphB gene product from a PCB degrading bacterium, Pseudomonas putida OU83, FEMS Microbiology Letters, 154; 317-24.

Khan, A.A., Wang, R. F., Nawaz, M. S., Cerniglia, C. E., (1997), Nucleotide sequence of the genes encoding cis-biphenyl dihydrodiol dehydrogenase (bphB) and the expression of an active recombinant His-tagged bphB gene product from a PCB degrading bacterium, Pseudomonas putida QU83, FEMS Microbiol Lett., 154; 317-324.

Khan, A.A., Wang, R.F., Nawaz, M.S., Cao, W.W., Cerniglia, C.E., Purification of 2,3-dihydroxybiphenyl 1,2-dioxygenase from Pseudomonas putida OU83 and characterization of the gene (bphC), Appl. Envir. Microbiol. 62: 1825-1830

Kimura, N., Nishi, A., Goto, M., Furukawa, K., (1997), Functional analyses of a variety of chimeric dioxygenases constructed from two biphenyl dioxygenases that are similar structurally but different functionally, J Bacteriol, 179; 3936-43.
Kumumaru, T., Suenaga, H., Mitsuoka, M., Furukawa, K., (1998), Enhanced degradation of polychlorinated biphenyls by directed evolution of biphenyl dioxygenase, Nature Biotechnology, 16; 663.

Lau, P.C.K., Labbe, D., Garnon, J., Bergeron, H., Wang, Y., (1995), Regulatory genes as biomonitoring tools and use of insertion sequences for strain identification, J. Cell. Biochem, Suppl. 21A; 49

Lloyd Jones, G., Ogden, R.C., Williams, P.A., (1995), Inactivation of 2,3-dihydroxybiphenyl-1,2-dioxygenase from Pseudomonas sp. strain CB406 by 3,4-dihydroxybiphenyl (4-phenylcatechol), Biodegradation, 6; 11-17.

Lobos, J.H., (1995), Isolation and characterization of novel PCB-degrading bacteria unable to grow on biphenyl, Abstr. Gen. Meet. Am. Soc. Microbiol, 95 Meet; 400.

Maltseva, O., Tsoi, T., Quensen, J. F., Fukuda, M., Tiedje, J.M., (1998), Aerobic degradation of the polychlorinated biphenyls which accumulate during anaerobic degradation of Aroclor, Abstr. Gen. Meet. Am. Soc. Microbiol, 98 Meet., 429-30.

Master, E.R., Mohn, W.M., (1998), Psychrotolerant bacteria isolated from arctic soil that degrade polychlorinated biphenyls at low temperatures, Applied and Environmental Microbiology, 64; 4823.

McKay, D.B., Seeger, M., Zielinski, M., Hofer, B., Timmis, K.N., (1997), Heterologous expression of biphenyl dioxygenase-encoding genes from a gram-positive broad-spectrum polychlorinated biphenyl degrader and characterization of chlorobiphenyl oxidation by the gene products, J. Bacteriol. 179: 1924-1930.

Mondello, F.J., (1989), Cloning and expression in Escherichia coli of Pseudomonas strain LB400 genes encoding polychlorinated biphenyl degradation, J. Bacteriol, 171; 1725-32.

Mondello, F.J., Turcich, M.P., Lobos, J.H., Erickson, B.D., (1997), Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation. Applied and Environmental Microbiology, 63; 3096.

Mondello, F.J., (1989), Cloning and expression in Escherichia coli of Pseudomonas strain LB400 genes encoding polychlorinated biphenyl degradation, J. Bacteriol, 171:3:1725- 1732.

Mondello, F.J., (1991), Comparing the ability of recombinant and naturally-occurring bacteria to degrade polychlorinated biphenyls on soil, Abstr. Gen. Meet. Am. Soc. Microbiol., 91 Meet.; 285

Mondello, F.J., Bopp, L. H., (1987), Genetic and cell-free studies of PCB biodegradation in Pseudomonas putida LB400, Biotech. USA, 171-81.

Mukerjee-Dhar, G., Hatta, T., Shimura, M., Kimbara, K., (1998), Analysis of changes in congener selectivity during PCB degradation by Burkholdaria sp. Strain TSN101 with increasing concentrations of PCB and characterization of the bphBCD genes and gene products, .Arch Microbiol. 169; 61-70.

Munthali, M.T., Timmis, K.N., Diaz, E., (1996), Restricting the dispersal of

recombinant DNA: design of a contained biological catalyst. Bio/Technology, 14; 189-91.

Nishazaki, T., Masai, E., Fukuda, M., (1998), Characterization of class-I extradiol-dioxygenase from a strong PCB degrader Rhodococcus sp. strain RHA1, Abstr. Gen. Meet. Am. Soc. Microbiol, 98 Meet., 427.

Potrawfke, T., Loehnert, T.H., Timmis, K.N., Wittich, R.M., (1998), Mineralization of low-chlorinated biphenyls by Burkholderia sp. strain LB400 and by 2 membered consortium upon directed interspecies transfer of chlorocatechol pathway genes, Appl. Microbiol. Biotechnol, 50; 440-46

Potrawfke, T., Löhnert, T.H., Timmis, K.N., Wittich, R.M., (1998), Mineralization of low-chlorinated biphenyls by Burkholderia sp. strain LB400 and by a two-membered consortium upon directed interspecies transfer of chlorocatechol pathway genes, Appl. Micro and Bio. 50; 440-446.

Reineke, W., (1998), Development of a hybrid strain for the mineralization of chloroaromatics by patchwork assembly, Annual Review of microbiology, Palo Alto,

Roberts, L., (1987), Discovering microbes with a taste for PCBs, Science, 237; 975-77.

Rothmel, R.K., Degrading polyaromatic and other hydrophobic organic compounds in soil, etc, Lawrenceville, NJ, USA

Seah, S.Y.K., Terracina, G., Bolin, J.T., Riebel, P., Snieckus, V., Eltis, L.D., (1998), Purification and Preliminary Characterization of a Serine Hydrolase Involved in the Microbial Degradation of Polychlorinated Biphenyls, J. Biol. Chem. 273: 22943-22949.

Seeger, M., Timmis, K.N., Hofer, B., (1995), Conversion of chlorobiphenyls into phenylhexadienoates and benzoates by the enzymes of the upper pathway for polychlorobiphenyl degradation encoded by the bph locus of Pseudomonas sp. strain LB400, Applied and Environmental Microbiology, 61; 2654

Seeger, M., Timmis, K.N., Hofer, B., (1995), Degradation of chlorobiphenyls catalyzed by the bph-encoded biphenyl-2,3-dioxygenase and biphenyl-2,3-dihydrodiol-2,3-dehydrogenase of Pseudomonas sp. LB400, FEMS Microbiology Letters, 133; 259-64.

Seeger, M., Timmis, K.N., Hofer, B., (1998), Bacterial pathways for the degradation of polychlorinated biphenyls, Oceanographic Literature Review, 45; 1036-37

Seeger,M., Zielinski, M., Timmis, K.N., and Hofer, B. (1999). Regiospecificity of Dioxygenation of Di- to Pentachlorobiphenyls and Their Degradation to Chlorobenzoates by the *bph*-Encoded Catabolic Pathway of *Burkholderia* sp. Strain LB400. Appl. Environ. Microbiol. 65: 3614-3621.

Soni, B.K., Phreykz, U.E., Kelley, R.L., Srivastava, V.J., (1996), Isolation,

characterization and PCB degradation studies of bacteria from PCB-contaminated soil, Abstr. Gen. Meet. Am. Soc. Microbiol, 96 Meet; 420

Spence, E.L., Kawamukai, M., Sanvoisin, J., Braven, H., Bugg, T.D.H., (1996), Catechol dioxygenases from Escherichia coli (MhpB) and Alcaligenesn eutrophus (MpcI): sequence analysis and biochemical properties of a third family of extradiol dioxygenases, Journal of Bacteriology, 178; 5249

Suenaga, H., Nishi, A., Watanabe, T., Sakai, M., Furukawa, K., (1999), Engineering a Hybrid Pseudomonad to Acquire 3,4-Dioxygenase Activity for Polychlorinated Biphenyls, J Bioscience and Bioengeneering, 87; 430-35.

Turcich, M.T., Erickson, B.D., Mondello, F.J., (1995), Functional comparisons and site-directed mutagenesis of genes involved in polychlorinated biphenyl degradation, Abstr. Gen. Meet. Am. Soc. Microbiol, 95 Meet; 400.

Unterman, R., Brennan, R.E., Brooks, D.P., Mobley, J.B., McDermott, J.B., Schwartz, C.C., (1987), Bacterial degradation of PCBs in soil, In; Proceedings of the 194-National Meeting of Amercian Chemical Society, Division of Environmental Chemistry, 135-38.

Unterman, R., Brennan, M.J., Brooks, R.E., Mobley, D.P., McDermott, J.B., Schwartz, C.C., (1987), Bacterial degradation of PCB in soil Abstr. Pap. Am. Chem. Soc.,194 Meet.; ENVR49.

Unterman, R., D.L. Bedard, M.J. Brennan, L.H. Bopp, F.J. Mondello, R.E. Brooks, D.P. Mobley, J.B. McDermott, C.C. Schwartz, D.K. Dietrich, G.S. Omenn, (1988), Biological approaches for polychlorinated biphenyl degradation. Environmental biotechnology: reducing risks from environmental chemicals through biotechnology, Reducing risks from environmental chemicals through biotechnology, 253-279.

Unterman, R., F.J. Mondello, M.J. Brennan, R.E. Brooks, D.P. Mobley, J.B. McDermott, and C.C. Schwartz, (1987), Bacterial treatment of PCB-contaminated soils. Prospects for the application of recombinant DNA technology, In: Proceedings of the second international conference on new frontiers for hazardous waste management. 591;259-264.

Van Dyke, M.I., Lee, H., Trevors, J. T., (1996), Survival of luxAB-marked Alcaligenes eutrophus H850 in PCB-contaminated soil and sediment, J Chem Technol Biotechnol, 65; 115-22.

Williams, W.A., (1993), The use of PCR to detect and enumerate aerobic polychlorobiphenyl (PCB) degrading organisms, Abstr. Gen. Meet. Am. Soc. Microbiol., 93 Meet.; 400.

Wong, W.P., Sterling, J., Yates, J.R., (1995), Instability of the bph cluster of PCB degrading bacteria related to Pseudomonas sp. LB400, Abstr. Gen. Meet. Am. Soc. Microbiol, 95 Meet; 506.

Yates, J.R., Mondello, F.J., (1989), Sequence similarities in the genes encoding

polychlorinated biphenyl degradation by Pseudomonas strain LB400 and alcaligenes eutrophus H850, Bacteriology,171; 1733-35.

APPENDIX

The sections of the appendix are numbered to match the main sections of the dossier.

Appendix B1
Technical details relating to the *lac* genes inserted in *P. fluorescens* F113lacZY.

Strain F113::lacZYrif: Details on gene products and DNA sequence.
Note that only *lacZ* and *lacY* are present in the GMO.

```
LOCUS       ECOLAC       7477 bp    DNA             BCT       05-MAY-1993
DEFINITION  E.coli lactose operon with lacI, lacZ, lacY and lacA genes.
ACCESSION   J01636 J01637 K01483 K01793
NID         g146575
KEYWORDS    acetyltransferase; beta-D-galactosidase; galactosidase; lac operon;
            lac repressor protein; lacA gene; lacI gene; lacY gene; lacZ gene;
            lactose permease; mutagenesis; palindrome; promoter region;
            thiogalactoside acetyltransferase.
SOURCE      Escherichia coli DNA; mRNA; clone lambda-h80dlac DNA; clone puk217;
            pgm8 (see comment).
  ORGANISM  Escherichia coli
            Eubacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae;
            Escherichia.
REFERENCE   1  (bases 1243 to 1266)
  AUTHORS   Gilbert,W. and Maxam,A.
  TITLE     The nucleotide sequence of the lac operator
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 70, 3581-3584 (1973)
  MEDLINE   74055539
REFERENCE   2  (bases 1246 to 1308)
  AUTHORS   Maizels,N.M.
  TITLE     The nucleotide sequence of the lactose messenger ribonucleic acid
            transcribed from the UV5 promoter mutant of Escherichia coli
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 70, 3585-3589 (1973)
  MEDLINE   74055540
REFERENCE   3  (sites)
  AUTHORS   Gilbert,W., Maizels,N. and Maxam,A.
  TITLE     Sequences of controlling regions of the lactose operon
  JOURNAL   Cold Spring Harb. Symp. Quant. Biol. 38, 845-855 (1974)
  MEDLINE   74174501
REFERENCE   4  (sites)
  AUTHORS   Gilbert,W., Gralla,J., Majors,A.J. and Maxam,A.
  TITLE     Lactose operator sequences and the action of lac repressor
  JOURNAL   (in) Sund,H. and Blauer,G. (Eds.);
            PROTEIN-LIGAND INTERACTIONS: 193-207;
            Walter de Gruyter, New York (1975)
REFERENCE   5  (bases 1146 to 1282)
  AUTHORS   Dickson,R.C., Abelson,J.N., Barnes,W.M. and Reznikoff,W.S.
  TITLE     Genetic regulation: The lac control region
  JOURNAL   Science 187, 27-35 (1975)
  MEDLINE   75048325
REFERENCE   6  (bases 1227 to 1271)
  AUTHORS   Gilbert,W., Maxam,A. and Mirzabekov,A.
  TITLE     Contacts between the lac repressor and DNA revealed by methylation
  JOURNAL   (in) Kjeldgaard,N.C. and Maaloe,O. (Eds.);
            CONTROL OF RIBOSOME SYNTHESIS: 138-143;
            Academic Press, New York (1976)
REFERENCE   7  (sites)
  AUTHORS   Marians,K.J. and Wu,R.
  TITLE     Structure of the lactose operator
  JOURNAL   Nature 260, 360-363 (1976)
  MEDLINE   76150089
REFERENCE   8  (bases 1242 to 1268)
  AUTHORS   Heyneker,H.L., Shine,J., Goodman,H.M, Boyer,H.W., Rosenberg,J.,
            Dickerson,R.E., Narang,S.A., Itakura,K., Lin,S. and Riggs,A.D.
  TITLE     Synthetic lac operator is functional in vivo
  JOURNAL   Nature 263, 748-752 (1976)
  MEDLINE   77056376
REFERENCE   9  (sites)
  AUTHORS   Dickson,R.C., Abelson,J.N., Johnson,P., Reznikoff,W.S. and
            Barnes,W.M.
  TITLE     Nucleotide sequence changes produced by mutations in the lac
            promoter of Escherichia coli
  JOURNAL   J. Mol. Biol. 111, 65-75 (1977)
  MEDLINE   77168230
REFERENCE   10 (bases 51 to 264)
  AUTHORS   Steege,D.A.
```

TITLE 5'-terminal nucleotide sequence of Escherichia coli lactose repressor mRNA: Features of translational initiation and reinitiation sites
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 74, 4163-4167 (1977)
MEDLINE 78052881

REFERENCE 11 (bases 1 to 81)
AUTHORS Calos,M.P.
TITLE DNA sequence for a low-level promoter of the lac repressor and an 'up' promoter mutation
JOURNAL Nature 274, 762-765 (1978)
MEDLINE 78246990

REFERENCE 12 (bases 49 to 1161)
AUTHORS Farabaugh,P.J.
TITLE Sequence of the lacI gene
JOURNAL Nature 274, 765-769 (1978)
MEDLINE 78246991

REFERENCE 13 (sites)
AUTHORS Miller,J.H., Coulondre,C. and Farabaugh,P.J.
TITLE Correlation of nonsense sites in the lacI gene with specific codons in the nucleotide sequence
JOURNAL Nature 274, 770-775 (1978)
MEDLINE 78246992

REFERENCE 14 (sites)
AUTHORS Calos,M.P. and Miller,J.H.
TITLE DNA sequence alteration resulting from a mutation impairing promoter function in the lac repressor gene
JOURNAL Mol. Gen. Genet. 178, 225-227 (1980)
MEDLINE 80209248

REFERENCE 15 (bases 4306 to 5804)
AUTHORS Buechel,D.E., Gronenborn,B. and Mueller-Hill,B.
TITLE Sequence of the lactose permease gene
JOURNAL Nature 283, 541-545 (1980)
MEDLINE 80120651

REFERENCE 16 (sites)
AUTHORS Miller,J.H., Calos,M.P. and Galas,D.J.
TITLE Genetic and sequencing studies of the specificity of transposition into the lac region of E. coli
JOURNAL Cold Spring Harb. Symp. Quant. Biol. 45, 243-257 (1981)
MEDLINE 82049502

REFERENCE 17 (sites)
AUTHORS Chenchick,A., Beabealashvilli,R.S. and Mirzabekov,A.
TITLE Topography of interaction of Escherichia coli RNA polymerase subunits with lac UV5 promoter
JOURNAL FEBS Lett. 128, 46-50 (1981)
MEDLINE 82004657

REFERENCE 18 (sites)
AUTHORS Betz,J.L. and Sadler,J.R.
TITLE Variants of a cloned synthetic lactose operator: I. A palindromic dimer lactose operator derived from one strand of the cloned 40-base pair operator
JOURNAL Gene 13, 1-12 (1981)
MEDLINE 81213459

REFERENCE 19 (sites)
AUTHORS Sadler,J.R. and Tecklenburg,M.
TITLE Cloning and characterization of the natural lactose operator
JOURNAL Gene 13, 13-23 (1981)
MEDLINE 81213463

REFERENCE 20 (sites)
AUTHORS Betz,J.L. and Sadler,J.R.
TITLE Variants of a cloned synthetic lactose operator: II. Chloramphenicol-resistant revertants retaining a lactose opeator in the CAT gene of plasmid pBR325
JOURNAL Gene 15, 187-200 (1981)
MEDLINE 82051311

REFERENCE 21 (sites)
AUTHORS Calos,M.P. and Miller,J.H.
TITLE The DNA sequence change resulting from the I-Q1 mutation, which greatly increases promoter strength
JOURNAL Mol. Gen. Genet. 183, 559-560 (1981)
MEDLINE 82147746

REFERENCE 22 (sites)
AUTHORS Mieschendahl,M., Buechel,D.E., Bocklage,H. and Mueller-Hill,B.
TITLE Mutations in the lacY gene of Escherichia coli define functional organization of lactose permease
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 78, 7652-7656 (1981)
MEDLINE 82150928

REFERENCE 23 (sites)

AUTHORS Russell,D.R. and Bennett,G.N.
TITLE Construction and analysis of in vivo activity of E. coli promoter hybrids and promoter mutants that alter the -35 to -10 spacing
JOURNAL Gene 20, 231-243 (1982)
MEDLINE 83158761
REFERENCE 24 (sites)
AUTHORS Horowitz,H. and Platt,T.
TITLE A termination site for lacI transcription is between the CAP site and the lac promoter
JOURNAL J. Biol. Chem. 257, 11740-11746 (1982)
MEDLINE 83007251
REFERENCE 25 (sites)
AUTHORS Klein,R.D. and Wells,R.D.
TITLE Effects of neighboring DNA homopolymers on the biochemical and physical properties of the Escherichia coli lactose promoter: I. Cloning and characterization studies
JOURNAL J. Biol. Chem. 257, 12954-12961 (1982)
MEDLINE 83030833
REFERENCE 26 (bases 1183 to 1291)
AUTHORS Weiher,H. and Schaller,H.
TITLE Segment-specific mutagenesis: Extensive mutagenesis of a lac promoter/operator element
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 79, 1408-1412 (1982)
MEDLINE 82174608
REFERENCE 27 (sites)
AUTHORS Van Dyke,M.W. and Dervan,P.B.
TITLE Footprinting with MPE-Fe(II). Complementary-strand analyses of distamycin- and actinomycin-binding sites on heterogeneous DNA
JOURNAL Cold Spring Harb. Symp. Quant. Biol. 47, 347-353 (1983)
MEDLINE 83233528
REFERENCE 28 (bases 1287 to 4364)
AUTHORS Kalnins,A., Otto,K., Ruether,U. and Mueller-Hill,B.
TITLE Sequence of the lacZ gene of Escherichia coli
JOURNAL EMBO J. 2, 593-597 (1983)
MEDLINE 84028567
REFERENCE 29 (sites)
AUTHORS Cone,K.C., Sellitti,M.A. and Steege,D.A.
TITLE Lac repressor mRNA transcription terminates in vivo in the lac control region
JOURNAL J. Biol. Chem. 258, 11296-11304 (1983)
MEDLINE 83291093
REFERENCE 30 (sites)
AUTHORS Sadler,J.R., Sasmor,H. and Betz,J.L.
TITLE A perfectly symmetric lac operator binds the lac repressor very tightly
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 80, 6785-6789 (1983)
MEDLINE 84070714
REFERENCE 31 (sites)
AUTHORS Glickman,B.W. and Ripley,L.S.
TITLE Structural intermediates of deletion mutagenesis: A role for palindromic DNA
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 81, 512-516 (1984)
MEDLINE 84119517
REFERENCE 32 (sites)
AUTHORS Spassky,A., Kirkegaard,K. and Buc,H.
TITLE Changes in the DNA structure of the lac UV5 promoter during formation of an open complex with Escherichia coli RNA polymerase
JOURNAL Biochemistry 24, 2723-2731 (1985)
MEDLINE 85280412
REFERENCE 33 (sites)
AUTHORS Straney,D.C. and Crothers,D.M.
TITLE Intermediates in transcription initiation from the E. coli lac UV5 promoter
JOURNAL Cell 43, 449-459 (1985)
MEDLINE 86079527
REFERENCE 34 (sites)
AUTHORS Looman,A.C., de Gruyter,M., Vogelaar,A. and van Knippenberg,P.H.
TITLE Effects of heterologous ribosomal binding sites on the transcription and translation of the lacZ gene of Escherichia coli
JOURNAL Gene 37, 145-154 (1985)
MEDLINE 86031346
REFERENCE 35 (sites)
AUTHORS Mandecki,W., Goldman,R.A., Powell,B.S. and Caruthers,M.H.
TITLE Lac up-promoter mutants with increased homology to the consensus promoter sequence
JOURNAL J. Bacteriol. 164, 1353-1355 (1985)
MEDLINE 86059235

```
REFERENCE   36 (sites)
  AUTHORS   Malamy,M.H., Rahaim,P.T., Hoffman,C.S., Baghdoyan,D., O'Connor,M.B.
            and Miller,J.F.
  TITLE     A frameshift mutation at the junction of an IS1 insertion within
            lacZ restores beta-galactosidase activity via formation of an
            active lacZ-IS1 fusion protein
  JOURNAL   J. Mol. Biol. 181, 551-555 (1985)
  MEDLINE   85210885
REFERENCE   37 (bases 5646 to 7477)
  AUTHORS   Hediger,M.A, Johnson,D.F., Nierlich,D.P. and Zabin,I.
  TITLE     DNA sequence of the lactose operon: The lacA gene and the
            transcriptional termination region
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 82, 6414-6418 (1985)
  MEDLINE   86016712
COMMENT     [3]  sites; UV5 mRNA transcripts and operator mutants. [(in)
            Sund,H. and Blauer,G. (eds.);Protein-Ligand Interactions:
            193-207;Walter de]  sites; operator mutational analysis. [7]
            sites; S1 and mung bean nuclease action on operator DNA. [9]
            sites; class I, II and III promoter mutant analysis. [13]  sites;
            lacI mutant analysis.
            [16]  sites; Tn5, Tn9 and Tn10 insertion sites in lac region. [14]
            sites; lacI promoter mutation UJ177.
            [18]  sites; palindromic dimer operator;.
            [19]  sites; natural operator sequence.
            [20]  sites; operator mutational analysis.
            [21]  sites; lacI-Q deletion.
            [17]  sites; RNA polymerase UV5 promoter interaction. [22]  sites;
            lacY mutational analysis.
            [24]  sites; lacI-promoted transcription termination. [25]  sites;
            wt and UV5 promoter sequence studies. [23]  sites; UV5 promoter
            mutational analysis.
            [30]  sites; perfectly symmetric operator sequence. [29]  sites;
            lacI mRNA termination site.
            [27]  sites; distamycin and actinomycin binding to promoter. [31]
            sites; lacI deletion studies.
            [35]  sites; promoter mutational studies.
            [33]  sites; DNAase I studies with promoter sequence. [34]  sites;
            ribosomal binding and translation initiation for lacZ. [36]  sites;
            insertion sequence IS1 integration in lacZ;. [32]  sites; DNAase I
            studies with promoter.
            [1] first reports a 27 bp operator(sites 1240-1266) with two-fold
            symmetries; the operator has also been defined to be bases
            1246-1266 or bases 1239-1273 [8]. [(in) Kjeldgaard,N.C. and Maaloe,
            O.(eds);Control of ribosome synthesis: 138-143;A] explores the
            ability of lac
            repressor protein to affect methylation of operator DNA.  [8]
            argues that DNA on both sides of the 21 bp operator (bases
            1246-1266) affects repressor binding but that the sequences of this
            DNA are probably not critical. [5] gives a larger sequence known as
            the promoter-operator region for the wild-type, whereas [2] and
            [26] give portions of this region for the mutant strain UV5. Within
            the promoter region, bases 1162-1199 are identified as the
            catabolite gene activator protein binding site (cap) and bases
            1200-1245 are the RNA polymerase interaction site. [10] reports a
            sequence for the 5'end of the lacI (repressor) gene and discusses
            restart in mutant strains. [11] presents a sequence for the lacI
            promoter region and identifies an I-Q mutation which enhances lacI
            transcription approximately ten-fold. [12] gives a complete
            sequence
            for lacI which agrees with the known lac repressor sequence. [26]
            examines the promoter-operator region in the UV5 strain (lac109)
            and studies 23 mutant derivatives of this sequence. This sequence
            agrees with known protein sequences for the lacZ, lacY and lacA
            enzymes. [15] notes that the fMet codon is not present
            for lacA and suggests that the 'ttg' codon (5727-5729), which
            immediately precedes the mature N-terminal asparagine codon, is the
            start codon. The cds for lacZ, lacY and lacA are included on a
            single mRNA transcript.
            Complete source information:
            Escherichia coli DNA [1],[(in) Kjeldgaard,N.C. and Maaloe,O.(eds);
            Control of ribosome synthesis: 138-143;A],[8],[12],[26]; mRNA [2],
            [5],[10]; clone
            lambda-h80dlac DNA [11],[15]; clone puk217 [28]; pgm8 [37].
FEATURES             Location/Qualifiers
     source          1..7477
                     /organism="Escherichia coli"
                     /db_xref="taxon:562"
```

```
mutation        16
                /note="c in wild-type; t in 'up' promoter mutant I-Q [11]"
mRNA            51..1230
                /note="lacI (repressor) mRNA; preferred in vivo 3' end
                [12],[29]"
gene            79..1161
                /gene="lacI"
CDS             79..1161
                /gene="lacI"
                /note="lac repressor protein (gtg start codon)"
                /codon_start=1
                /transl_table=11
                /db_xref="PID:g146576"
                /translation="MKPVTLYDVAEYAGVSYQTVSRVVNQASHVSAKTREKVEAAMAE
                LNYIPNRVAQQLAGKQSLLIGVATSSLALHAPSQIVAAIKSRADQLGASVVVSMVERS
                GVEACKAAVHNLLAQRVSGLIINYPLDDQDAIAVEAACTNVPALFLDVSDQTPINSII
                FSHEDGTRLGVEHLVALGHQQIALLAGPLSSVSARLRLAGWHKYLTRNQIQPIAEREG
                DWSAMSGFQQTMQMLNEGIVPTAMLVANDQMALGAMRAITESGLRVGADISVVGYDDT
                EDSSCYIPPSTTIKQDFRLLGQTSVDRLLQLSQGQAVKGNQLLPVSLVKRKTTLAPNT
                QTASPRALADSLMQLARQVSRLESGQ"
misc_signal     1162..1199
                /note="cap protein binding site"
variation       1183..1186
                /note="ttag in wild-type; aatt in strain UV5 [26]"
mutation        1209..1211
                /note="gct in wild-type; gt in mutant l305 [5]"
mutation        1212
                /note="t in wild-type; a in mutant l241 [5]"
mutation        1230
                /note="c in wild-type; a in mutant p-r-1a [5]"
variation       1237..1238
                /note="gt in wild-type; aa in strain UV5 [26]"
mutation        1242..1245
                /note="gtgg in wild-type; ttca in synthetic operator [8]"
misc_signal     1246..1266
                /note="lac repressor protein binding site"
mRNA            1246..>4358
                /note="lacZ mRNA [2],[5]"
mutation        1267..1268
                /note="tc in wild-type; tg in synthetic operator [8]"
variation       1282..1291
                /note="ctatgaccat in wild-type; gatccggcca in strain UV5
                [26]"
gene            1284..4358
                /gene="lacZ"
CDS             1284..4358
                /gene="lacZ"
                /note="beta-d-galactosidase"
                /codon_start=1
                /transl_table=11
                /db_xref="PID:g146577"
                /translation="MTMITDSLAVVLQRRDWENPGVTQLNRLAAHPPFASWRNSEEAR
                TDRPSQQLRSLNGEWRFAWFPAPEAVPESWLECDLPEADTVVVPSNWQMHGYDAPIYT
                NVTYPITVNPPFVPTENPTGCYSLTFNVDESWLQEGQTRIIFDGVNSAFHLWCNGRWV
                GYGQDSRLPSEFDLSAFLRAGENRLAVMVLRWSDGSYLEDQDMWRMSGIFRDVSLLHK
                PTTQISDFHVATRFNDDFSRAVLEAEVQMCGELRDYLRVTVSLWQGETQVASGTAPFG
                GEIIDERGGYADRVTLRLNVENPKLWSAEIPNLYRAVVELHTADGTLIEAEACDVGFR
                EVRIENGLLLLNGKPLLIRGVNRHEHHPLHGQVMDEQTMVQDILLMKQNNFNAVRCSH
                YPNHPLWYTLCDRYGLYVVDEANIETHGMVPMNRLTDDPRWLPAMSERVTRMVQRDRN
                HPSVIIWSLGNESGHGANHDALYRWIKSVDPSRPVQYEGGGADTTATDIICPMYARVD
                EDQPFPAVPKWSIKKWLSLPGETRPLILCEYAHAMGNSLGGFAKYWQAFRQYPRLQGG
                FVWDWVDQSLIKYDENGNPWSAYGGDFGDTPNDRQFCMNGLVFADRTPHPALTEAKHQ
                QQFFQFRLSGQTIEVTSEYLFRHSDNELLHWMVALDGKPLASGEVPLDVAPQGKQLIE
                LPELPQPESAGQLWLTVRVVQPNATAWSEAGHISAWQQWRLAENLSVTLPAASHAIPH
                LTTSEMDFCIELGNKRWQFNRQSGFLSQMWIGDKKQLLTPLRDQFTRAPLDNDIGVSE
                ATRIDPNAWVERWKAAGHYQAEAALLQCTADTLADAVLITTAHAWQHQGKTLFISRKT
                YRIDGSGQMAITVDVEVASDTPHPARIGLNCQLAQVAERVNWLGLGPQENYPDRLTAA
                CFDRWDLPLSDMYTPYVFPSENGLRCGTRELNYGPHQWRGDFQFNISRYSQQQLMETS
                HRHLLHAEEGTWLNIDGFHMGIGGDDSWSPSVSAEFQLSAGRYHYQLVWCQK"
gene            4410..5663
                /gene="lacY"
CDS             4410..5663
                /gene="lacY"
                /note="lactose permease"
                /codon_start=1
                /transl_table=11
                /db_xref="PID:g146578"
```

```
                     /translation="MYYLKNTNFWMFGLFFFFYFFIMGAYFPFFPIWLHDINHISKSD
                     TGIIFAAISLFSLLFQPLFGLLSDKLGLRKYLLWIITGMLVMFAPFFIFIFGPLLQYN
                     ILVGSIVGGIYLGFCFNAGAPAVEAFIEKVSRRSNFEFGRARMFGCVGWALCASIVGI
                     MFTINNQFVFWLGSGCALILAVLLFFAKTDAPSSATVANAVGANHSAFSLKLALELFR
                     QPKLWFLSLYVIGVSCTYDVFDQQFANFFTSFFATGEQGTRVFGYVTTMGELLNASIM
                     FFAPLIINRIGGKNALLLAGTIMSVRIIGSSFATSALEVVILKTLHMFEVPFLLVGCF
                     KYITSQFEVRFSATIYLVCFCFFKQLAMIFMSVLAGNMYESIGFQGAYLVLGLVALGF
                     TLISVFTLSGPGPLSLLRRQVNEVA"
     gene            5727..6338
                     /gene="lacA"
     CDS             5727..6338
                     /gene="lacA"
                     /note="thiogalactoside acetyltransferase (ttg start
                     codon)"
                     /codon_start=1
                     /transl_table=11
                     /db_xref="PID:g551814"
                     /translation="MNMPMTERIRAGKLFTDMCEGLPEKRLRGKTLMYEFNHSHPSEV
                     EKRESLIKEMFATVGENAWVEPPVYFSYGSNIHIGRNFYANFNLTIVDDYTVTIGDNV
                     LIAPNVTLSVTGHPVHHELRKNGEMYSFPITIGNNVWIGSHVVINPGVTIGDNSVIGA
                     GSIVTKDIPPNVVAAGVPCRVIREINDRDKHYYFKDYKVESSV"
BASE COUNT     1739 a   1991 c   2004 g   1743 t
ORIGIN      HindII site [Nature 274, 762-765 (1978)].
        1 gacaccatcg aatggcgcaa aacctttcgc ggtatggcat gatagcgccc ggaagagagt
       61 caattcaggg tggtgaatgt gaaaccagta acgttatacg atgtcgcaga gtatgccggt
      121 gtctcttatc agaccgtttc ccgcgtggtg aaccaggcca gccacgtttc tgcgaaaacg
      181 cgggaaaaag tggaagcggc gatggcggag ctgaattaca ttcccaaccg cgtggcacaa
      241 caactggcgg gcaaacagtc gttgctgatt ggcgttgcca cctccagtct ggccctgcac
      301 gcgccgtcgc aaattgtcgc ggcgattaaa tctcgcgccg atcaactggg tgccagcgtg
      361 gtggtgtcga tggtagaacg aagcggcgtc gaagcctgta aagcggcggt gcacaatctt
      421 ctcgcgcaac gcgtcagtgg gctgatcatt aactatccgc tggatgacca ggatgccatt
      481 gctgtggaag ctgcctgcac taatgttccg gcgttatttc ttgatgtctc tgaccagaca
      541 cccatcaaca gtattatttt ctcccatgaa gacggtacgc gactgggcgt ggagcatctg
      601 gtcgcattgg gtcaccagca aatcgcgctg ttagcgggcc cattaagttc tgtctcggcg
      661 cgtctgcgtc tggctggctg gcataaatat ctcactcgca atcaaattca gccgatagcg
      721 gaacgggaag gcgactggag tgccatgtcc ggttttcaac aaaccatgca aatgctgaat
      781 gagggcatcg ttcccactgc gatgctggtt gccaacgatc agatggcgct gggcgcaatg
      841 cgcgccatta ccgagtccgg gctgcgcgtt ggtgcggata tctcggtagt gggatacgac
      901 gataccgaag acagctcatg ttatatcccg ccgtcaacca ccatcaaaca ggattttcgc
      961 ctgctggggc aaaccagcgt ggaccgcttg ctgcaactct ctcagggcca ggcggtgaag
     1021 ggcaatcagc tgttgcccgt ctcactggtg aaaagaaaaa ccaccctggc gcccaatacg
     1081 caaaccgcct ctccccgcgc gttggccgat tcattaatgc agctggcacg acaggtttcc
     1141 cgactggaaa gcgggcagtg agcgcaacgc aattaatgtg agttagctca ctcattaggc
     1201 accccaggct ttacacttta tgcttccggc tcgtatgttg tgtggaattg tgagcggata
     1261 acaatttcac acaggaaaca gctatgacca tgattacgga ttcactggcc gtcgttttac
     1321 aacgtcgtga ctgggaaaac cctggcgtta cccaacttaa tcgccttgca gcacatcccc
     1381 ctttcgccag ctggcgtaat agcgaagagg cccgcaccga tcgcccttcc caacagttgc
     1441 gcagcctgaa tggcgaatgg cgctttgcct ggtttccggc accagaagcg gtgccggaaa
     1501 gctggctgga gtgcgatctt cctgaggccg atactgtcgt cgtcccctca aactggcaga
     1561 tgcacggtta cgatgcgccc atctacacca acgtaaccta tcccattacg gtcaatccgc
     1621 cgtttgttcc cacggagaat ccgacgggtt gttactcgct cacatttaat gttgatgaaa
     1681 gctggctaca ggaaggccag acgcgaatta ttttgatgg cgttaactcg gcgtttcatc
     1741 tgtggtgcaa cgggcgctgg gtcggttacg gccaggacag tcgtttgccg tctgaatttg
     1801 acctgagcgc atttttacgc gccggagaaa accgcctcgc ggtgatggtg ctgcgttgga
     1861 gtgacggcag ttatctggaa gatcaggata tgtggcggat gagcggcatt ttccgtgacg
     1921 tctcgttgct gcataaaccg actacacaaa tcagcgattt ccatgttgcc actcgcttta
     1981 atgatgattt cagccgcgct gtactggagg ctgaagttca gatgtgcggc gagttgcgtg
     2041 actacctacg ggtaacagtt tctttatggc agggtgaaac gcaggtcgcc agcggcaccg
     2101 cgcctttcgg cggtgaaatt atcgatgagc gtggtggtta tgccgatcgc gtcacactac
     2161 gtctgaacgt cgaaaacccg aaactgtgga gcgccgaaat cccgaatctc tatcgtgcgg
     2221 tggttgaact gcacaccgcc gacggcacgc tgattgaagc agaagcctgc gatgtcggtt
     2281 tccgcgaggt gcggattgaa aatggtctgc tgctgctgaa cggcaagccg ttgctgattc
     2341 gaggcgttaa ccgtcacgag catcatcctc tgcatggtca ggtcatggat gagcagacga
     2401 tggtgcagga tatcctgctg atgaagcaga acaactttaa cgccgtgcgc tgttcgcatt
     2461 atccgaacca tccgctgtgg tacacgctgt gcgaccgcta cggcctgtat gtggtggatg
     2521 aagccaatat tgaaacccac ggcatggtgc caatgaatcg tctgaccgat gatccgcgct
     2581 ggctaccggc gatgagcgaa cgcgtaacgc gaatggtgca gcgcgatcgt aatcacccga
     2641 gtgtgatcat ctggtcgctg gggaatgaat caggccacgg cgctaatcac gacgcgctgt
     2701 atcgctggat caaatctgtc gatccttccc gcccggtgca gtatgaaggc ggcggagccg
     2761 acaccacggc caccgatatt atttgcccga tgtacgcgcg cgtggatgaa gaccagccct
     2821 tcccggctgt gccgaaatgg tccatcaaaa aatggctttc gctacctgga gagacgcgcc
```

2881 cgctgatcct ttgcgaatac gcccacgcga tgggtaacag tcttggcggt ttcgctaaat
2941 actggcaggc gtttcgtcag tatccccgtt tacagggcgg cttcgtctgg gactgggtgg
3001 atcagtcgct gattaaatat gatgaaaacg gcaacccgtg gtcggcttac ggcggtgatt
3061 ttggcgatac gccgaacgat cgccagttct gtatgaacgg tctggtcttt gccgaccgca
3121 cgccgcatcc agcgctgacg gaagcaaaac accagcagca gtttttccag ttccgtttat
3181 ccgggcaaac catcgaagtg accagcgaat acctgttccg tcatagcgat aacgagctcc
3241 tgcactggat ggtggcgctg gatggtaagc cgctggcaag cggtgaagtg cctctggatg
3301 tcgctccaca aggtaaacag ttgattgaac tgcctgaact accgcagccg gagagcgccg
3361 ggcaactctg gctcacagta cgcgtagtgc aaccgaacgc gaccgcatgg tcagaagccg
3421 ggcacatcag cgcctggcag cagtggcgtc tggcggaaaa cctcagtgtg acgctccccg
3481 ccgcgtccca cgccatcccg catctgacca ccagcgaaat ggatttttgc atcgagctgg
3541 gtaataagcg ttggcaattt aaccgccagt caggctttct ttcacagatg tggattggcg
3601 ataaaaaaca actgctgacg ccgctgcgcg atcagttcac ccgtgcaccg ctggataacg
3661 acattggcgt aagtgaagcg acccgcattg accctaacgc ctgggtcgaa cgctggaagg
3721 cggcgggcca ttaccaggcc gaagcagcgt tgttgcagtg cacggcagat acacttgctg
3781 atgcggtgct gattacgacc gctcacgcgt ggcagcatca ggggaaaacc ttatttatca
3841 gccggaaaac ctaccggatt gatggtagtg gtcaaatggc gattaccgtt gatgttgaag
3901 tggcgagcga tacaccgcat ccggcgcgga ttggcctgaa ctgccagctg gcgcaggtag
3961 cagagcgggt aaactggctc ggattagggc cgcaagaaaa ctatcccgac cgccttactg
4021 ccgcctgttt tgaccgctgg gatctgccat tgtcagacat gtataccccg tacgtcttcc
4081 cgagcgaaaa cggtctgcgc tgcgggacgc gcgaattgaa ttatggccca caccagtggc
4141 gcggcgactt ccagttcaac atcagccgct acagtcaaca gcaactgatg gaaaccagcc
4201 atcgccatct gctgcacgcg gaagaaggca catggctgaa tatcgacggt ttccatatgg
4261 ggattggtgg cgacgactcc tggagcccgt cagtatcggc ggaattccag ctgagcgccg
4321 gtcgctacca ttaccagttg gtctggtgtc aaaaataata ataaccgggc aggccatgtc
4381 tgcccgtatt tcgcgtaagg aaatccatta tgtactattt aaaaaacaca aacttttgga
4441 tgttcggttt attcttttttc ttttactttt ttatcatggg agcctacttc ccgtttttcc
4501 cgatttggct acatgacatc aaccatatca gcaaaagtga tacgggtatt atttttgccg
4561 ctatttctct gttctcgcta ttattccaac cgctgtttgg tctgctttct gacaaactcg
4621 ggctgcgcaa atacctgctg tggattatta ccggcatgtt agtgatgttt gcgccgttct
4681 ttatttttat cttcgggcca ctgttacaat acaacatttt agtaggatcg attgttggtg
4741 gtatttatct aggcttttgt tttaacgccg gtgcgccagc agtagaggca tttattgaga
4801 aagtcagccg tcgcagtaat ttcgaatttg gtcgcgcgcg gatgtttggc tgtgttggct
4861 gggcgctgtg tgcctcgatt gtcggcatca tgttcaccat caataatcag tttgttttct
4921 ggctgggctc tggctgtgca ctcatcctcg ccgttttact ctttttcgcc aaaacggatg
4981 cgccctcttc tgccacggtt gccaatgcgg taggtgccaa ccattcggca tttagcctta
5041 agctggcact ggaactgttc agacagccaa aactgtggtt tttgtcactg tatgttattg
5101 gcgtttcctg cacctacgat gtttttgacc aacagtttgc taatttcttt acttcgttct
5161 ttgctaccgg tgaacagggt acgcgggtat ttggctacgt aacgacaatg ggcgaattac
5221 ttaacgcctc gattatgttc tttgcgccac tgatcattaa tcgcatcggt gggaaaaacg
5281 ccctgctgct ggctggcact attatgtctg tacgtattat tggctcatcg ttcgccacct
5341 cagcgctgga agtggttatt ctgaaaacgc tgcatatgtt tgaagtaccg ttcctgctgg
5401 tgggctgctt taaatatatt accagccagt ttgaagtgcg tttttcagcg acgatttatc
5461 tggtctgttt ctgcttcttt aagcaactgg cgatgatttt tatgtctgta ctggcgggca
5521 atatgtatga aagcatcggt ttccagggcg cttatctggt gctgggtctg gtggcgctgg
5581 gcttcacctt aatttccgtg ttcacgctta gcggccccgg cccgctttcc ctgctgcgtc
5641 gtcaggtgaa tgaagtcgct taagcaatca atgtcggatg cggcgcgacg cttatccgac
5701 caacatatca taacggagtg atcgcattga acatgccaat gaccgaaaga ataagagcag
5761 gcaagctatt taccgatatg tgcgaaggct taccggaaaa aagacttcgt gggaaaacgt
5821 taatgtatga gtttaatcac tcgcatccat cagaagttga aaaaagagaa agcctgatta
5881 aagaaatgtt tgccacggta ggggaaaacg cctgggtaga accgcctgtc tatttctctt
5941 acggttccaa catccatata ggccgcaatt tttatgcaaa tttcaattta accattgtcg
6001 atgactacac ggtaacaatc ggtgataacg tactgattgc acccaacgtt actctttccg
6061 ttacgggaca ccctgtacac catgaattga gaaaaaacgg cgagatgtac tcttttccga
6121 taacgattgg caataacgtc tggatcggaa gtcatgtggt tattaatcca ggcgtcacca
6181 tcggggataa ttctgttatt ggcgcgggta gtatcgtcac aaaagacatt ccaccaaacg
6241 tcgtggcggc tggcgttcct tgtcgggtta ttcgcgaaat aaacgaccgg gataagcact
6301 attatttcaa agattataaa gttgaatcgt cagtttaaat tataaaaatt gcctgatacg
6361 ctgcgcttat caggcctaca agttcagcga tctacattag ccgcatccgg catgaacaaa
6421 gcgcaggaac aagcgtcgca tcatgcctct ttgacccaca gctgcggaaa acgtactggt
6481 gcaaaacgca gggttatgat catcagccca acgacgcaca gcgcatgaaa tgcccagtcc
6541 atcaggtaat tgccgctgat actacgcagc acgccagaaa accacggggc aagcccggcg
6601 atgataaaac cgattccctg cataaacgcc accagcttgc cagcaatagc cggttgcaca
6661 gagtgatcga gcgccagcag caaacagagc ggaaacgcgc cgcccagacc taacccacac
6721 accatcgccc acaataccgg caattgcatc ggcagccaga taaagccgca gaaccccacc
6781 agttgtaaca ccagcgccag cattaacagt ttgcgccgat cctgatggcg agccatagca
6841 ggcatcagca aagctcctgc ggcttgccca agcgtcatca atgccagtaa ggaaccgctg
6901 tactgcgcgc tggcaccaat ctcaatatag aaagcgggta accaggcaat caggctggcg

```
     6961 taaccgccgt taatcagacc gaagtaaaca cccagcgtcc acgcgcgggg agtgaatacc
     7021 acgcgaaccg gagtggttgt tgtcttgtgg gaagaggcga cctcgcgggc gctttgccac
     7081 caccaggcaa agagcgcaac aacggcaggc agcgccacca ggcgagtgtt tgataccagg
     7141 tttcgctatg ttgaactaac cagggcgtta tggcggcacc aagcccaccg ccgcccatca
     7201 gagccgcgga ccacagcccc atcaccagtg gcgtgcgctg ctgaaaccgc cgtttaatca
     7261 ccgaagcatc accgcctgaa tgatgccgat ccccacccca ccaagcagtg cgctgctaag
     7321 cagcagcgca ctttgcgggt aaagctcacg catcaatgca ccgacggcaa tcagcaacag
     7381 actgatggcg acactgcgac gttcgctgac atgctgatga agccagcttc cggccagcgc
     7441 cagcccgccc atggtaacca ccggcagagc ggtcgac
//
```

Appendix B2
Technical information relating to the *bph* genes inserted in *P. fluorescens* F113rifpcb:
More details on protein and DNA sequences

```
LOCUS       PSEBPHA      5700 bp    DNA             BCT       18-JUL-1997
DEFINITION  Pseudomonas sp. LB400 biphenyl dioxygenase (bphA), biphenyl
            dioxygenase (bphE), biphenyl dioxygenase (bphF) and biphenyl
            dioxygenase (bphG)s, complete cds, and dihydrodiol dehydrogenase
            (bphB), partial cds.
ACCESSION   M86348
NID         g349602
KEYWORDS    .
SOURCE      Burkholderia sp. LB400.
  ORGANISM  Burkholderia sp. LB400
            Eubacteria; Proteobacteria; beta subdivision; Burkholderia.
REFERENCE   1  (bases 1 to 5700)
  AUTHORS   Erickson,B.D. and Mondello,F.J.
  TITLE     Nucleotide sequencing and transcriptional mapping of genes
encoding
            biphenyl dioxygenase, a multicomponent PCB-degrading enzyme in
            pseudomonas strain LB400
  JOURNAL   J. Bacteriol. 174, 2903-2912 (1992)
  MEDLINE   92234948
REFERENCE   2  (bases 1 to 5700)
  AUTHORS   Erickson,B.D.
  JOURNAL   Unpublished (1993)
FEATURES             Location/Qualifiers
     source          1..5700
                     /organism="Burkholderia sp. LB400"
                     /strain="LB400"
                     /db_xref="taxon:36873"
     mRNA            523..>5700
                     /note="alternate transcription site"
                     /evidence=experimental
     CDS             611..1348
                     /note="ORF 0"
                     /codon_start=1
                     /transl_table=11
                     /db_xref="PID:g151083"

/translation="MNARTPNSLTMGGDKSFADASPVPIANVRSLIEATFQRLRADIV

EGRLAAGSRLAIEDLKSRYEVSGGTVREALSLLVANNLVQTQAQRGFHVTPMSLDDMR

DLAATRIALECEALRQSVLNGDAEWEARVVSSYHRLSLLDERTMRDPVHLFNQWEQAN

RDFHEALISACSSAWTQRFLSILYLQMERYRRLTAMHNRPARNVHEEHLALRDSALAR
                     DAERCTELLRMHIESSISVVRQFGLLR"
     mRNA            1370..>5700
                     /note="alternate transcription site"
                     /evidence=experimental
     gene            1439..2818
                     /gene="bphA"
     CDS             1439..2818
                     /gene="bphA"
                     /standard_name="iron sulfur protein"
                     /note="large subunit of the terminal dioxygenase
                     component"
                     /codon_start=1
                     /transl_table=11
                     /product="biphenyl dioxygenase"
                     /db_xref="PID:g151084"

/translation="MSSAIKEVQGAPVKWVTNWTPEAIRGLVDQEKGLLDPRIYADQS

LYELELERVFGRSWLLLGHESHVPETGDFLATYMGEDPVVMVRQKDKSIKVFLNQCRH
```

```
RGMRICRSDAGNAKAFTCSYHGWAYDIAGKLVNVPFEKEAFCDKKEGDCGFDKAEWGP

LQARVATYKGLVFANWDVQAPDLETYLGDARPYMDVMLDRTPAGTVAIGGMQKWVIPC

NWKFAAEQFCSDMYHAGTTTHLSGILAGIPPEMDLSQAQIPTKGNQFRAAWGGHGSGW

YVDEPGSLLAVMGPKVTQYWTEGPAAELAEQRLGHTGMPVRRMVGQHMTIFPTCSFLP

TFNNIRIWHPRGPNEIEVWAFTLVDADAPAEIKEEYRRHNIRNFSAGGVFEQDDGENW

VEIQKGLRGYKAKSQPLNAQMGLGRSQTGHPDFPGNVGYVYAEEAARGMYHHWMRMMS
                     EPSWATLKP"
     gene            2936..3502
                     /gene="bphE"
     CDS             2936..3502
                     /gene="bphE"
                     /standard_name="iron sulfur protein"
                     /note="small subunit of the terminal dioxygenase
                     component"
                     /codon_start=1
                     /transl_table=11
                     /product="biphenyl dioxygenase"
                     /db_xref="PID:g151085"

/translation="MTNPSPHFFKTFEWPSKAAGLELQNEIEQFYYREAQLLDHRAYE

AWFALLDKDIHYFMPLRTNRMIREGELEYSGDQDLAHFDETHETMYGRIRKVTSDVGW

AENPPSRTRHLVSNVIVKETATPDTFEVNSAFILYRNRLERQVDIFAGERRDVLRRAD
                     NNLGFSIAKRTILLDASTLLSNNLSMFF"
     CDS             3540..3959
                     /note="iron sulfur protein; ORF 1"
                     /codon_start=1
                     /transl_table=11
                     /db_xref="PID:g151086"

/translation="MKNARLFLIAIGVFYIINLIGTLPFSTLGLFGRMYPGVELHVGA

PIFTLLQDAWAVVGLQLGAIGAVALWGARDPGRYRAVIPVVIATEVVDGLWDFYSIVW
                     SHEALWFGLVTLVIHVLWIGWGLHAWRALASKSLRTL"
     gene            4034..4363
                     /gene="bphF"
     CDS             4034..4363
                     /gene="bphF"
                     /standard_name="iron sulfur protein"
                     /note="ferredoxin component of biphenyl dioxygenase"
                     /codon_start=1
                     /transl_table=11
                     /product="biphenyl dioxygenase"
                     /db_xref="PID:g349603"

/translation="MKFTRVCDRRDVPEGEALKVESGGTSVAIFNVDGELFATQDRCT

HGDWSLSDGGYLEGDVVECSLHMGKFCVRTGKVKSPPPCEALKIFPIRIEDNDVLVDF
                     EAGYLAP"
     gene            4360..5586
                     /gene="bphG"
     CDS             4360..5586
                     /gene="bphG"
                     /standard_name="iron sulfur protein"
                     /note="reductase component of biphenyl dioxygenase"
                     /codon_start=1
                     /transl_table=11
                     /product="biphenyl dioxygenase"
                     /db_xref="PID:g349604"

/translation="MIDTIAIIGAGLAGSTAARALRAQGYEGRIHLLGDESHQAYDRT
```

```
TLSKTVLAGEQPEPPAILDSAWYASAHVDVQLGRRVSCLDLANRQIQFESGAPLAYDR

LLLATGARARRMAIRGGDLAGIHTLRDLADSQALRQALQPGQSLVIVGGGLIGCEVAT

TARKLSVHVTILEAGDELLVRVLGHRTGAWCRAELERMGVRVERNAQAARFEGQGQVR

AVICADGRRVPADVVLVSIGAEPADELARAAGIACARGVLVDATGATSCPEVFAAGDV

AAWPLRQGGQRSLETYLNSQMEAEIAASAMLSQPVPAPQVPTSWTEIAGHRIQMIGDA

EGPGEIVVRGDAQSGQPIVLLRLLDGCVEAATAINATREFSVATRLVGTRVSVSAEQL
                    QDVGSNLRDLLKAKPN"
     gene           5633..5700
                    /gene="bphB"
     CDS            5633..>5700
                    /gene="bphB"
                    /note="amino terminus of the dihyrodiol dehydrogenase"
                    /codon_start=1
                    /transl_table=11
                    /product="dihydrodiol dehydrogenase"
                    /db_xref="PID:g151089"
                    /translation="MKLKGEAVLITGGASGLGRALVX"
BASE COUNT     1107 a   1678 c   1801 g   1114 t
ORIGIN
        1 gaattccgcg aagaacacca gttggccgtg cggcgtggcc gccgcacctt cgtcccagcg
       61 cacgtgcatg cgcccgccca gcgtgtccac gatctgcggc tggacgggct catggcctgc
      121 ccttggacgc gccgccagcg ccttgcgtct tgcttcaccc atcgggtgag tgtgccaaaa
      181 ccgcgcaaag ccgcaactgg actggctcag cgcccgatca cggtcgagca actgccggat
      241 ttaggatcat ccaaaacgat gtgggagcgg ctgaaacccg tcagggatcg cccgaaccgc
      301 aagtcgtgtc aagttccacc tgtacctgat cctggatttg tacagccgca agatcgtggg
      361 tgccgaggtg cacgacagcg acgaatctgt gcatgccgtg catctggtgc ggcgcaccgc
      421 actggccgag ggcatcgcag cgatggacac caaacccgta ctaagacctt tcctagggtt
      481 ttcactcagg tatggatgtc gaatgtcgat aataatatca acattatgac gagccacttg
      541 ctatcgaagc ctgattcagt ccgcgctgaa atgtggggaa attttctgta atgaccaggt
      601 gagaaatgca atgaatgcga gaactccaaa cagcctcacc atgggaggcg acaagtcatt
      661 tgcggatgcg tcgccggtgc ccatcgcgaa cgtgcgcagc ctgattgagg cgacgtttca
      721 gcggttgcgg gcggacatcg tggaggggcg actcgctgct ggctccagac tcgccattga
      781 agatctcaag tcgcgctatg aggttagcgg cgggaccgtg cgggaggcgc tttccctgct
      841 ggtggccaat aacctggtgc agacacaggc ccagcgtgga tttcatgtga cccccatgtc
      901 tctggatgac atgcgtgatc tggccgccac gcggatcgcg cttgagtgcg aagcgctgcg
      961 ccaaagtgtg ctgaatggcg acgccgaatg ggaggcgcgg gttgtcagtt cgtatcaccg
     1021 gctctcgctc ctcgacgagc gcacgatgcg cgatccggtt cacttgttca atcagtggga
     1081 gcaagccaac cgcgactttc acgaagccct gatctcagcc tgttcatccg cctggaccca
     1141 gcggttcctg tctatcctgt atttgcaaat ggagcgctac cgccgattga cggcgatgca
     1201 caaccggcct gccagaaacg tgcacgagga gcatctggcg cttcgtgaca gcgcgctcgc
     1261 gcgtgatgcc gagcgctgta ctgagttgtt gcggatgcat attgagtcat caatttcggt
     1321 ggttcggcaa ttcggtttgt tgcggtgacg ccaacatcgc aggtatcttt tttgtgtgat
     1381 ggaagaggtt ggtatttttt ccgccctgcc aagggcattt caacggagac gttaaatcat
     1441 gagttcagca atcaaagaag tgcagggagc ccctgtgaag tgggttacca attggacgcc
     1501 ggaggcgatc cgggggttgg tcgatcagga aaaagggctg cttgatccac gcatctacgc
     1561 cgatcagagt ctttatgagc tggagcttga gcgggttttt ggtcgctctt ggctgttact
     1621 tgggcacgag agtcatgtgc ctgaaaccgg ggacttcctg gccacttaca tgggcgaaga
     1681 tccggtggtt atggtgcgac agaaagacaa gagcatcaag gtgttcctga accagtgccg
     1741 gcaccgcggc atgcgtatct gccgctcgga cgccggcaac gccaaggctt tcacctgcag
     1801 ctatcacggc tgggcctacg acatcgccgg caagctggtg aacgtgccgt tcgagaagga
     1861 agccttttgc gacaagaaag aaggcgactg cggctttgac aaggccgaat ggggcccgct
     1921 ccaggcacgc gtggcaacct acaagggcct ggtctttgcc aactgggatg tgcaggcgcc
     1981 agacctggag acctacctcg gtgacgcccg cccctatatg gacgtcatgc tggatcgcac
     2041 gccggccggg actgtggcca tcggcggcat gcagaagtgg gtgattccgt gcaactggaa
     2101 gtttgccgcc gagcagttct gcagtgacat gtaccacgcc ggcaccacga cgcacctgtc
     2161 cggcatcctg gcgggcattc cgccggaaat ggacctctcc caggcgcaga tacccaccaa
     2221 gggcaatcag ttccgggccg cttggggcgg gcacggctcg ggctggtatg tcgacgagcc
     2281 gggctcactc ctggcggtga tgggccccaa ggtcacccag tactggaccg agggtccggc
     2341 tgccgagctt gcggaacagc gcctggggca caccggcatg ccggttcgac gcatggtcgg
     2401 ccagcacatg acgatcttcc cgacctgttc attcctgccc accttcaaca acatccggat
     2461 ctggcacccg cgtggtccca atgaaatcga ggtgtgggcc ttcaccctgg tcgatgccga
     2521 cgccccggcg gagatcaagg aagaatatcg ccggcacaac atccgcaact tctccgcagg
```

```
2581 cggcgtgttt gagcaggacg atggcgagaa ctgggtggag atccagaagg ggctacgtgg
2641 gtacaaggcc aagagccagc cgctcaatgc ccagatgggc ctgggtcggt cgcagaccgg
2701 tcaccctgat tttcctggca acgtcggcta cgtctacgcc gaagaagcgg cgcggggtat
2761 gtatcaccac tggatgcgca tgatgtccga gcccagctgg gccacgctca agccctgatc
2821 aagacgcaat cgttagatct gtcaaccgga agaattcaac atggtgggct ggacgtgcat
2881 gtgcagacgg cgcgccgagg ttccgtcccc tgatatttac ttggagataa ctgttatgac
2941 aaatccatcc ccgcattttt tcaaaacatt tgaatggcca agcaaggcgg ctggccttga
3001 gttgcagaac gagatcgagc agttctacta ccgcgaagcg cagttgcttg accaccgggc
3061 ctacgaggcc tggtttgccc tgctggacaa agatatccac tacttcatgc cgctgcgcac
3121 caatcgcatg atccgggagg gcgagctgga atattccggc gaccaggatt tagcccattt
3181 cgatgaaacc catgaaacca tgtacgggcg catccgcaag gtgacctcgg acgtgggctg
3241 ggcggagaac ccgccttccc gcacgcgcca cctggtctcc aacgtcatcg tcaaggagac
3301 ggccacgccg gataccttcg aggtcaattc cgcattcatc ctgtaccgca atcggcttga
3361 gcgccaggtc gacatcttcg cgggcgaacg ccgggacgtg ctgcgccgcg ccgacaacaa
3421 ccttggtttc agcatcgcca agcgcaccat cctgctcgac gccagtacct tgctgtcgaa
3481 caacctgagc atgttcttct agcccagcac gctgaaccgg cctcaatgag gatgctgcca
3541 tgaaaaatgc aagactgttt ttgatcgcca tcggcgtctt ctacatcatc aacctcattg
3601 gcacgcttcc cttcagcacg ttgggcttgt ttggcaggat gtatccaggc gtagaactgc
3661 acgtgggtgc gccgattttc accctgctgc aggatgcctg ggcggtggtc ggtctccagt
3721 tgggcgccat cggggccgtc gctttgtggg gcgcccgcga tccgggccgt tatcgggccg
3781 ttattccagt ggtcatcgca acggaagtgg tcgatggcct ctgggatttt tacagcatcg
3841 tgtggagcca cgaagccttg tggttcgggc ttgtcacgct ggtgatccat gtgctgtgga
3901 ttggctgggg cctgcatgcc tggcgtgccc tggcgtcgaa atcgctgagg acactttgaa
3961 ttactcttca gccaccaaca gtgactgttc gccccaggcg atttaaccct tttaactaat
4021 tacaagaagc gttatgaaat ttaccagagt ttgtgatcga agagatgtgc ccgaaggcga
4081 agccctgaag gtcgaaagtg gaggcacctc cgtcgcgatt ttcaatgtgg atggcgagct
4141 gttcgcaaca caggaccgct gcacccacgg cgactggtcc ctgtccgatg gcggctatct
4201 tgaaggtgac gtggtggaat gctcactgca catggggaag ttttgcgttc gcacgggcaa
4261 ggtcaaatca ccgccgccct gtgaggcact gaagatattt ccgatccgca tcgaagacaa
4321 tgacgtgctg gtcgacttcg aagccgggta tctggcgcca tgatcgacac catcgccatc
4381 atcggcgccg gcctggccgg ttcgacggct gcgcgcgcac tgcgcgccca gggatacgag
4441 gggcgcatcc acctgctcgg ggatgagtcg catcaggcct atgaccggac cacgctgtcc
4501 aagacggtgc tggcgggcga gcagcccgag ccgcctgcaa tcctggacag cgcctggtac
4561 gcatcggccc atgtggatgt ccagctcggg cgacgggtga gttgcctgga tctggccaac
4621 cgccagattc agtttgaatc gggcgccccg ctggcctacg accggctgct gctggccacc
4681 ggcgcgcgcg cccggcgcat ggcgattcgg ggtggcgacc tggcaggcat ccataccttg
4741 cgagacctcg ccgacagcca ggcgctgcgg caggcgctgc aaccgggcca gtcgctggtc
4801 atcgtcggcg gaggcctgat cggttgcgag gtggcgacca ccgcccgcaa gctgagtgtc
4861 catgtcacga ttctggaagc cggcgacgag ttgctggtgc gcgtgctggg tcaccggacc
4921 ggggcatggt gtcgggccga actggaacgc atgggtgtcc gcgtggagcg caatgcacag
4981 gccgcgcgct tcgaaggcca ggggcaggtg cgcgccgtga tctgcgccga cgggcgccgg
5041 gtgcccgccg atgtggtctt ggtcagcatt ggcgccgagc cggcggacga gctggcccgt
5101 gccgctggca tcgcctgcgc gcgcggcgtg ctggtcgacg ccaccggcgc cacctcgtgt
5161 ccagaggtgt tcgccgccgg tgacgtcgcc gcctggccgc tgcgtcaagg gggccagcgc
5221 tcgctggaga cctacctgaa cagccagatg gaggccgaaa tcgcggccag cgccatgttg
5281 agtcagcccg tgccggcgcc ccaggtgccg acctcgtgga cggagattgc aggccaccgc
5341 atccagatga ttggcgatgc cgaagggccc ggcgagatcg tcgtacgcgg cgacgcccag
5401 agcggccagc caatcgtgtt gctcaggctg cttgatggct gcgtcgaggc cgcgacggcg
5461 atcaatgcca ccagggaatt ttctgtggcg acccgactgg tcggcacccg ggtttctgtt
5521 tccgccgagc aactgcagga cgtcggctcg aacctgcggg atttactcaa agccaaaccg
5581 aattgatgcg catgaccggc gaatcgcttt aacaataaag gggattggaa aaatgaaact
5641 gaaaggtgaa gcggtactga tcacgggggg cgcctccgga ttagggcgcg cgctcgtgga
//
```

```
LOCUS       PSPBPH       1963 bp    DNA             BCT       02-MAY-1994
DEFINITION  Pseudomonas sp. genes bphB and bphC.
ACCESSION   X66122
NID         g397882
KEYWORDS    biphenyl degradation protein; bphB gene; bphC gene.
SOURCE      Pseudomonas sp.
  ORGANISM  Pseudomonas sp.
            Eubacteria; Proteobacteria.
REFERENCE   1  (bases 1 to 1963)
  AUTHORS   Hofer,B.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-JUN-1992) B. Hofer, G.B.F., Bereich Mikrobiologie,
            Mascheroder Weg 1, 3300 Braunschweig, FRG
REFERENCE   2  (bases 1 to 1963)
  AUTHORS   Hofer,B., Eltis,L.D., Dowling,D.N. and Timmis,K.N.
  TITLE     Genetic analysis of a Pseudomonas locus encoding a pathway for
            biphenyl/polychlorinated biphenyl degradation
  JOURNAL   Gene 130 (1), 47-55 (1993)
  MEDLINE   93345822
COMMENT     See also X66123 & M86348.
FEATURES             Location/Qualifiers
     source          1..1963
                     /organism="Pseudomonas sp."
                     /strain="LB400"
                     /db_xref="taxon:306"
                     /clone="E.coli SM10 (pDD5301)"
     gene            127..960
                     /gene="BphB"
     CDS             127..960
                     /gene="BphB"
                     /codon_start=1
                     /transl_table=11
                     /evidence=experimental
                     /product="Biphenyl-2,3-dihydro-2,3-diol dehydrogenase"
                     /db_xref="PID:g397883"
                     /db_xref="SWISS-PROT:P47227"

/translation="MKLKGEAVLITGGASGLGRALVDRFVAEGAKVAVLDKSAERLAE

LETDHGDNVLGIVGDVRSLEDQKQAASRCVARFGKIDTLIPNAGIWDYSTALVDLPEE

SLDAAFDEVFHINVKGYIHAVKACLPALVASRGNVIFTISNAGFYPNGGGPLYTAAKH

AIVGLVRELAFELAPYVRVNGVGSGGINSDLRGPSSLGMGSKAISTVPLADMLKSVLP

IGRMPEVEEYTGAYVFFATRGDAAPATGALLNYDGGLGVRGFFSGAGGNDLLEQLNIH
                     P"
     gene            982..1878
                     /gene="BphC"
     CDS             982..1878
                     /gene="BphC"
                     /codon_start=1
                     /transl_table=11
                     /evidence=experimental
                     /product="Biphenyl-2,3-diol-1,2 dioxygenase"
                     /db_xref="PID:g397884"
                     /db_xref="SWISS-PROT:P47228"

/translation="MSIRSLGYMGFAVSDVAAWRSFLTQKLGLMEAGTTDNGDLFRID

SRAWRIAVQQGEVDDLAFAGYEVADAAGLAQMADKLKQAGIAVTTGDASLARRRGVTG

LITFADPFGLPLEIYYGASEVFEKPFLPGAAVSGFLTGEQGLGHFVRCVPDSDKALAF

YTDVLGFQLSDVIDMKMGPDVTVPAYFLHCNERHHTLAIAAFPLPKRIHHFMLEVASL

DDVGFAFDRVDADGLITSTLGRHTNDHMVSFYASTPSGVEVEYGWSARTVDRSWVVVR
                     HDSPSMWGHKSVRDKAAARNKA"
```

```
BASE COUNT      387 a    555 c    616 g    405 t
ORIGIN
        1 cccgggtttc tgtttccgcc gagcaactgc aggacgtcgg ctcgaacctg cgggatttac
       61 tcaaagccaa accgaattga tgcgcatgac cggcgaatcg ctttaacaat aaaggggatt
      121 ggaaaaatga aactgaaagg tgaagcggta ctgatcacgg ggggcgcctc cggattaggg
      181 cgcgcgctcg tggaccgatt cgtggccgaa ggcgccaaag tggcggtgct cgacaagtcg
      241 gcagagcgcc ttgcagagct ggaaaccgat catggcgaca acgtgctcgg catcgtcggc
      301 gatgtgcgtt cactggaaga ccagaaacag gctgccagcc gctgcgtggc caggttcggg
      361 aaaatcgaca ccttgattcc caatgcaggc atctgggatt actcgacggc cttggtcgac
      421 ctgcccgaag agagcctcga tgccgcgttc gatgaggtct ttcacatcaa tgtcaagggt
      481 tatatccatg cagtgaaggc ctgcctgccg gccctggtcg ccagccgtgg caacgtgatc
      541 ttcacgatct ccaatgcggg cttctatccc aatggcggcg gccctcttta caccgcagcc
      601 aagcacgcca tcgtgggcct ggtgcgtgaa ctggcgttcg agctggcgcc atacgtgcgc
      661 gtcaacggcg tggggtcggg cggcatcaat agcgatctga gaggtccttc ctcgctggga
      721 atgggcagca aggcgatttc gaccgtaccg ctggccgaca tgctgaagtc ggtgctgccg
      781 attggtcgca tgcccgaggt cgaagagtac accggggcct atgtgttttt tgccacccga
      841 ggcgatgcgg cgcctgcgac cggggccttg ctgaactacg acggtggctt gggcgttcgt
      901 ggattcttct cgggtgccgg aggtaatgac ttgctcgaac agctgaatat ccatccctaa
      961 gcatcgacga aggagacagt aatgagcatc agaagtttgg gatacatggg gtttgcggtc
     1021 agcgacgtag ctgcttggcg ttcgtttctg acgcagaaac tgggcttgat ggaagcgggc
     1081 acgaccgaca acggcgacct gttccgcatc gattcgagag cctggcggat cgccgttcag
     1141 cagggcgagg ttgacgatct ggcctttgcc ggctacgagg tggccgatgc ggcagggctg
     1201 gcgcagatgg ctgacaagct caaacaggcc ggtatcgcag tcaccaccgg cgatgcctca
     1261 ttggccaggc gccgcggggt gaccggattg atcacctttg ccgacccgtt tggcctgccg
     1321 ttggaaattt actatggcgc cagcgaggtg ttcgaaaaac cgttcctgcc tggtgcggcc
     1381 gtgtcgggtt tcctgaccgg cgagcaaggg ctggggcatt tcgtgcgctg cgttccggat
     1441 tcggacaagg cgctggcgtt ttataccgac gtgctcggct tccagttgtc tgacgtcatc
     1501 gacatgaaaa tggggccgga cgtgacggtt cctgcgtact tcctgcactg caacgaacgc
     1561 caccacaccc tggcaattgc ggcattcccg ctgccaaaac gcattcatca cttcatgctc
     1621 gaagtcgcct cgctcgatga cgtcggcttt gcatttgatc gggttgacgc ggacggcttg
     1681 atcacctcca cgctggggcg ccacaccaat gaccacatgg tgtcgttcta tgcctcgacc
     1741 ccgtccggag tagaggtcga gtatggctgg agtgcccgta ccgttgaccg ctcctgggtt
     1801 gtggtgaggc acgacagtcc gagcatgtgg ggccacaagt ctgtgcgcga caaagcagct
     1861 gcgcgcaaca aagcatgaac aacaacctga atagcacggg aatccctgca tgaaacttta
     1921 ctacagccct ggtgcctgct ccctgtcacc gcacattgca ttg
//
```

Appendix D -- (Unpublished information):
Technical details on the survival of the genetically modified organisms.

Pseudomonas fluorescens strains F113::lacZY, F113rifpcb and F113pcblac3.1 were evaluated in terms of risk assessment, using plant-soil microcosm models.

F113::lacZY in contaminated soil from the release site:
Strain F113::lacZY was inoculated on willow cuttings, and the survival of the strain in the rhizosphere was followed over 2, 4 and 6 weeks in a contaminated and a non-contaminated soil. Parallel to this, two parallel sets of cuttings inoculated with the strain were treated with the herbicide round up. After the partial decay of the roots (three weeks after treatment), the number of culturable cells of F113::lacZY was measured in one set of the willows. In the other set, new cuttings were planted in the soil to see whether the strain was able to recolonise the roots of the plants (over three weeks of growth).
The results of the experiment indicate that the PCB contamination did influence the colonisation and survival of F113 in the rhizosphere of the plants. The colonisation of the strain in the contaminated soil was less than the colonisation in the non-contaminated soil (Fig. 1). Furthermore, there was a tendency for a better survival of the strain in the non-contaminated soil than in the contaminated soil after the round up treatment (Fig. 2). The recolonisation of the willow plants seemed to be the same in the two soils.
In the experiment, there were unfortunately some methodological problems, resulting in an overestimation of the cell density of *P. fluorescens* in the experimental soil. The reporter gene *LacZY* alone was found to be insufficient in facilitating a selective detection method, as a high number of the indiginous soil bacteria were able to metabolise the substrate X-gal (although this caused blue colouring of colonies at a distinctly lower intensity). This was probably due to other metabolising enzymes with a lower affinity for X-gal. Future work will, therefore, rely on a rifampicin-resistant mutant of F113::lacZY for reducing false positive background counts.

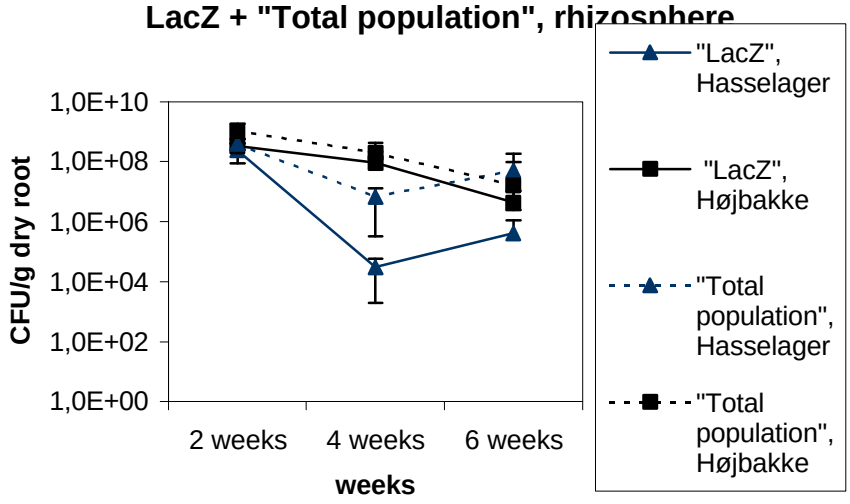


Figure 1: Survival of the strain F113::lacZY (abbreviated "lacZ") in a contaminated (Hasselager) and a non-contaminated (Højbakkegaard) soil after two, four and six weeks of incubation. Furthermore, the total population of fluorescent pseudomonads (SA medium) are shown. It can be seen that the survival of the strain is reduced by the contamination in the soil.

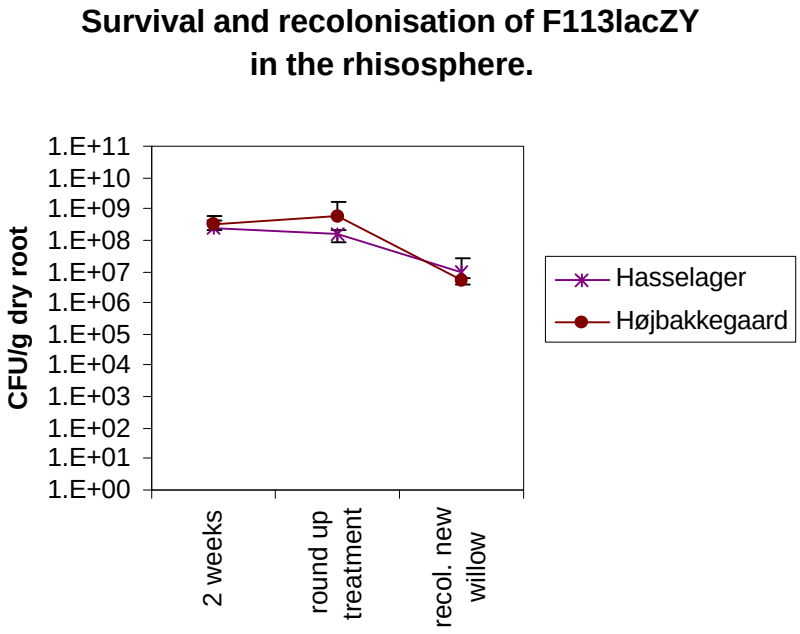


Figure 2: Survival and recolonisation of F113::lacZY in the rhizosphere of willow in a contaminated (Hasselager) and a non-contaminated (Højbakkegaard) soil. The data show the cell density of strain F113::lacZY after two weeks of incubation, after partial decay of the roots (because of round up treatment), and the recolonisation of a new willow planted in the round up treated rhizosphere. The results indicate that the strain is able to survive better in the non-contaminated soil than in the contaminated soil. There seems to be no difference in the recolonisation of the willow hereafter.

Nevertheless, even when including false positive counts, which (as backgound counts revealed) were only significant after 4 and 6 weeks, the data support an overall decreasing tendency of strain F113::lacZY's bacterial density in the experimental systems. When the initial inoculation was performed at ca. $1*10^8$ per system (corresponding to $1*10^6$ per g of soil), bacterial counts remained approximately constant or decreased over the first 4 weeks, whereas all final levels (after 6 weeks or after replanting with willow) were 2 to 3 orders of magnitude lower than initially inoculated.

<u>F113pcblac3.1 in soil piked with 4-PCB:</u>

Strain F113pcblac3.1 was evaluated for the ability to survive in the rhizosphere of willow and in bulk soil.

Cuttings of willow were inoculated with the strain at $8*10^9$ cells/cutting (= $8*10^9$ cells/microcosm system, corresponding to $0.84*10^6$ per g of soil). The inoculated cuttings were planted in a natural soil spiked with 4-PCB (200mg/kg). In the treatments for evaluating the survival of the strain in bulk soil without willow, $9*10^9$ cells (= $9*10^9$ cells/microcosm system, corresponding to $0.95*10^6$ per g of soil) were mixed into the soil. Every 14th day, over a period of 113 days, a triplicate of willows were extracted for the number of F113 in the systems. The results are shown in the following figure.

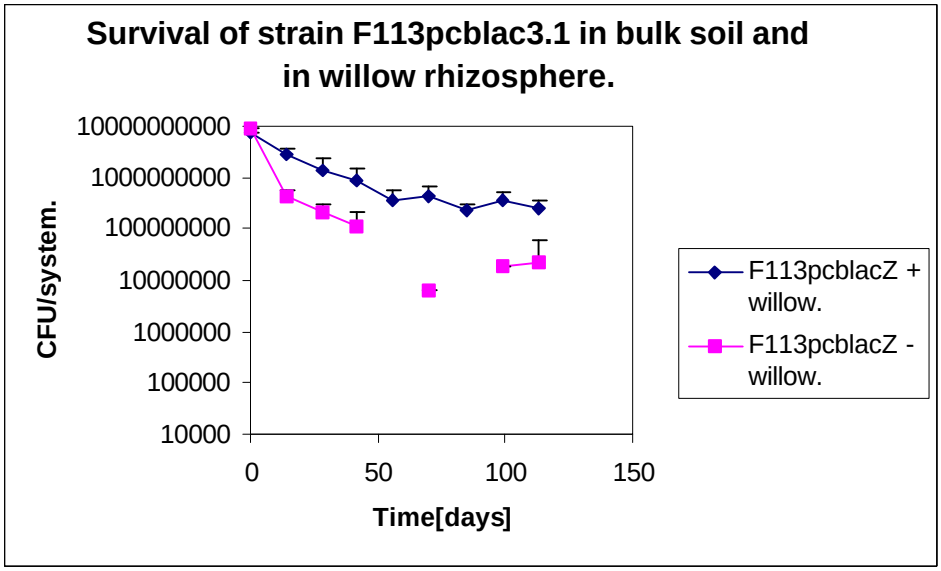


Fig. 3: Survival of F113pcblac3.1 (in the graph labelled F113pcblacZ) in soil microcosms with and without willow.

In both systems the number of bacteria decreased during the first 5 weeks after inoculation by more than 1 order of magnitude with willow, and by more than 2 orders of magnitude without willow. Hereafter, the number of bacteria seems to decrease at a slow rate during the remaining time of the experiment. For the systems consisting of willow-inoculated bacteria, the final level was about $3*10^8$ bacteria per system (corresponding to $3*10^6$ per g of soil). For the systems without the willow, the number is about $2*10^7$ bacteria per system (corresponding to $0.2*10^6$ per g of soil).
The experiment indicates, that the survival of *Pseudomonas fluorescens* F113pcblac3.1 in soil is good. The strain has an initial adaptation period for establishment, during which they rapidly decreases in number. Hereafter, the bacteria are present at an almost constant number in the systems. It is also indicated that the survival of the strain is enhanced about 10 times by the presence of willow.

<u>F113rifpcb in alfalfa microcosms:</u>
Strain F113rifpcb was evaluated for the ability to survive in the rhizosphere of alfalfa and in bulk soil.
Alfalfa seeds were inoculated with the strain at $1*10^6$ cells/seed (= $7*10^6$ cells/microcosm system, corresponding to $3*10^4$ per g of soil). The inoculated seeds were planted in a natural soil not spiked with PCB. Every 7th day, over a period of 4 weeks, a triplicate of alfalfa plants were extracted for the number of F113 on the root system and in the surrounding bulk soil. The Total Viable Count (TVC) of bacteria was also enumerated. The results are shown in the following figure.

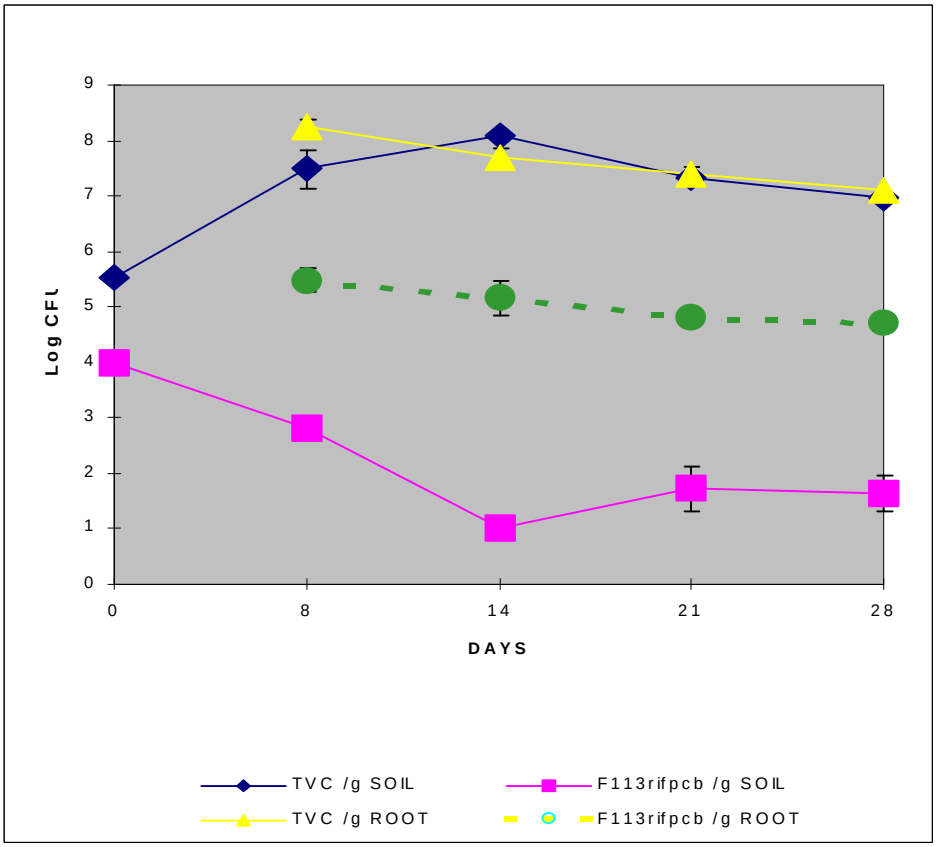


Fig. 4: Colonisation of alfalfa rhizosphere by *Pseudomonas fluorescens* F113rifpcb: Alginate encapsulated and inoculated alfalfa seeds in non-sterile soil microcosms. CFU, colony forming units; TVC, total viable count. (D. Dowling, unpublished data).

It can be seen, that the number of inoculated bacteria decreased during the 4 weeks of incubation by almost 1 order of magnitude in the root zone, and by more than 2 orders of magnitude in bulk soil. The experiment indicates, that the survival of *Pseudomonas fluorescens* F113rif pcb in soil is good, but limited. It is also indicated that the survival of the strain, after inoclation on seeds, is confined to the root zone, as its numbers in bulk soil rapidly decrease during the first two weeks after inoculation, reaching a constant plateau between 10^1 and 10^2 per g soil during these two two weeks.

Conclusions:

- Modified strains of *Pseudomonas fluorescens* F113 survive well in soil, i.e., their number of colony-forming units decreases only slowly. However their survival is not enhanced by the genetic modifications.
- There is no indication of growth of any of the strains exceeding the originally inoculated bacterial numbers.
- Plant removal reduces the strains' survival in soil.

Appendix E.

Results of soil analyses of the release site.

Analytical results: See table in the analysis report reproduced on the following page.

Sample codes: See text of the analysis report reproduced on the following page, and the map insert on it.

HYGIEJNELABORATORIET a.s
Plantagevej 37; DK-6270 Tønder; tlf: +45 74 72 47 00, fax: +45 74 72 57 83, www.hygilab.dk , e-mail: fh@hygilab.dk

Tønder den 31. maj 1999

DMU
Ulrich Karlson

1. sæt analyser i forbindelse med pileoprensning i Hasselager.

Den 30.04.99 blev det første sæt prøver udtaget, samtidig med at pilebeplantningen blev tilendebragt.

Prøveudtagning fandt sted som skitseret i forslag til analyseprogram med følgende ændringer:

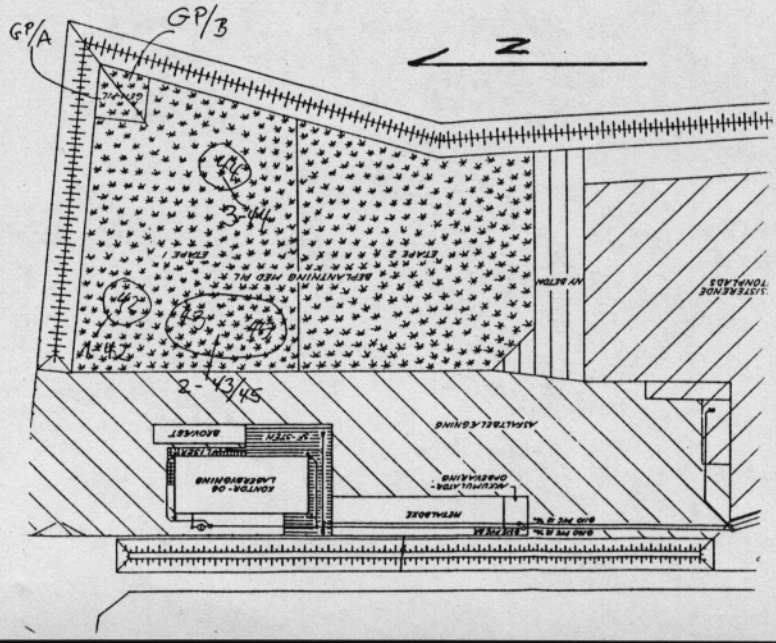
1. oprindelige prøvesteder 43 og 45 er slået sammen i stedet for 42 og 43. Mellem 42 og 43 var der oprindelig en kørevej, således at 43 og 45 mere udgør et sammenhængende område end 42 og 43.
2. der analyseres for oliekomponenter ved hj. af GC-FID, som ønsket af Århus Amt.

Nedenfor er der givet en oversigt over de fundne resultater:

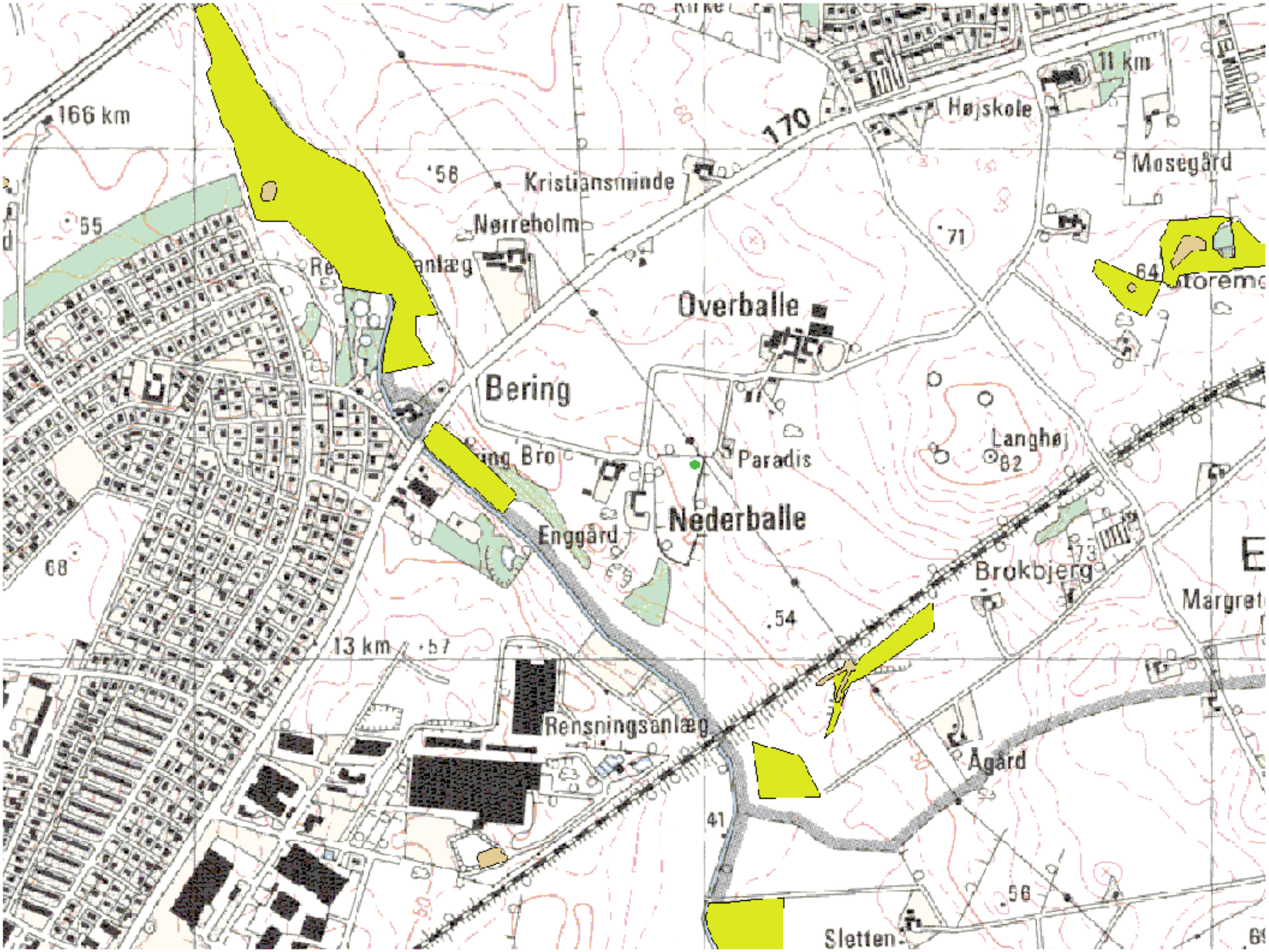
Parameter	GP/A	GP/B	1-42	2-43/45	3-44
tot. Kulbrinter i mg/kg tørstof	3.400	3.100	3.200	7.600	3.600
Benzen -"-	0,54	0,26	< 0,1	0,24	0,18
Toluen -"-	< 0,1	< 0,1	< 0,1	0,66	0,18
m+p Xylen -"-	< 0,1	< 0,1	< 0,1	1,6	0,10
o Xylen -"-	0,17	0,25	0,11	0,41	0,27
Ethylbenzen -"-	< 0,1	< 0,1	< 0,1	0,51	< 0,1
tot. Cyanid i mg/kg vådvægt			480	1.100	45
PCB 28 -"-	0,20	0,17			
PCB 52 -"-	0,26	0,21			
PCB 101 -"-	0,25	0,17			
PCB 118 -"-	0,16	0,099			
PCB 138 -"-	0,28	0,20			
PCB 153 -"-	0,19	0,14			
PCB 180 -"-	0,086	0,073			

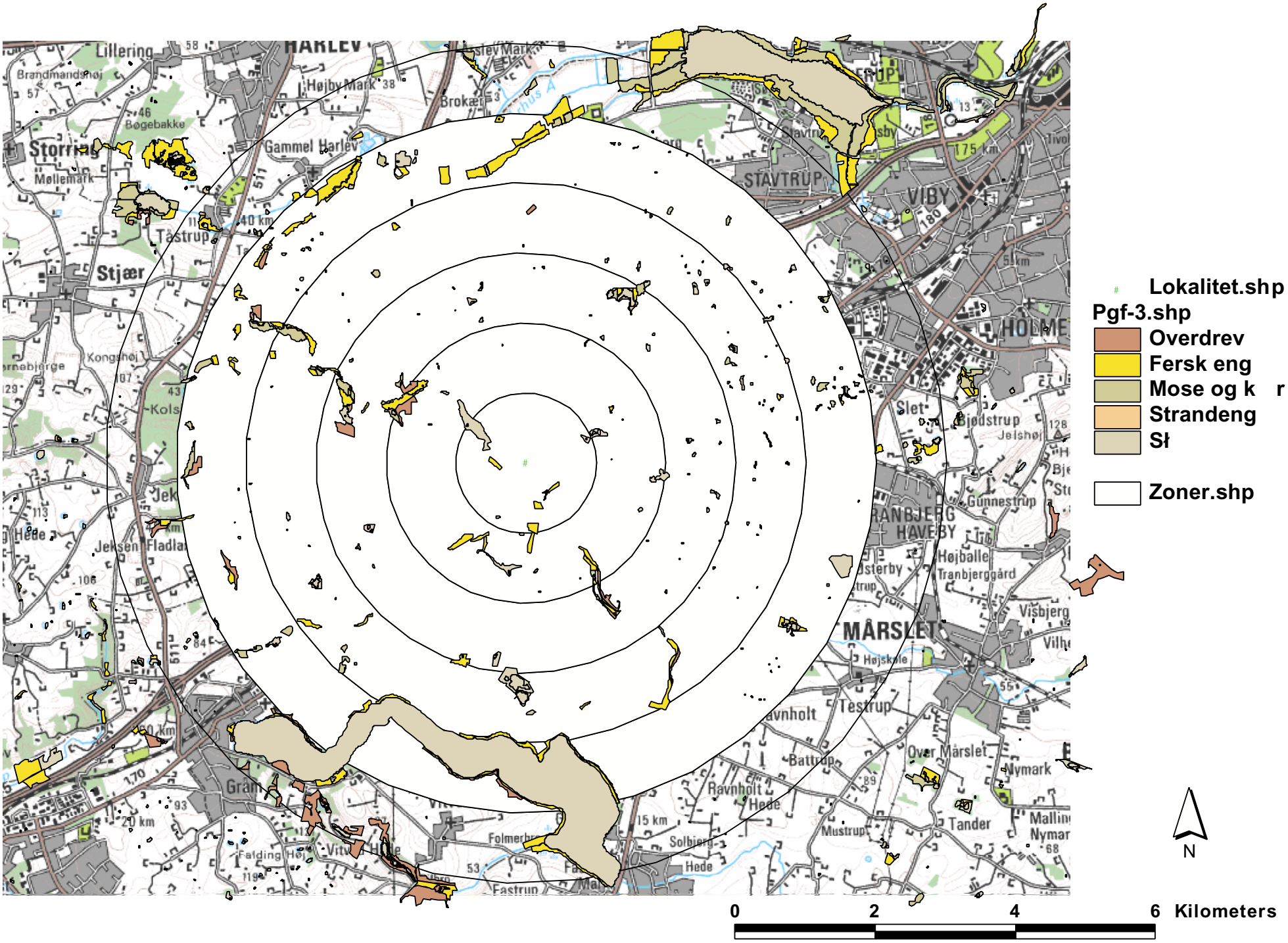
Med venlig hilsen

Fedder Hindrichsen



Two maps (see next 2 pages) showing the release site in relation to areas protected according to “Paragraf 3 Naturbeskyttelseslov”.


166 km
11 km
Højskole
170
Mosegård
Kristiansminde
Nørreholm
Overballe
Bering
Paradis
Langhøj
Nederballe
Enggård
Brokbjerg
13 km
Rensningsanlæg
Ågård
Sletten
Lokalitet
Paragraf 3 områder
Overdrev
Fersk eng
Mose og kær
Sø
0
500
1000 Meters
N


Lokalitet.shp
Pgf-3.shp
Overdrev
Fersk eng
Mose og k r
Strandeng
Sł
Zoner.shp
N
0
2
4
6 Kilometers

List of areas, protected according to "Paragraf 3 Naturbeskyttelseslov", in 0 to 6000 m distance from the release site. Sum of area in m^2. Note that the number of areas cannot be summed up across the whole list, as areas falling into more than one distance zone are counted more than once.

ZONE	0 - 1000 m	0 - 1000 m	1000 - 2000 m	1000 - 2000 m	2000 - 3000 m	2000 - 3000 m	3000 - 4000 m	3000 - 4000 m	4000 - 5000 m	4000 - 5000 m	5000 - 6000 m	5000 - 6000 m
	COUNT	SUM AREA	COUNT	SUM AREA	COUNT	SUM AREA	COUNT	SUM AREA	COUNT	SUM AREA	COUNT	SUM AREA
Overdrev			3	90274	7	161112	4	30298	8	164801	17	415348
Fersk eng	8	182967	17	370067	15	234977	19	521943	42	1157859	27	746714
Mose og kær			7	24523	14	130902	20	197261	28	345633	32	530098
Sø	4	4104	23	45413	45	108721	67	18711765	75	7594593	74	8973449

Appendix F.

Oplysninger leveret af Holger Pedersen, MST, landbrugs- og bioteknologikontor baseret på den oficielle rapport fra de kompetente myndigheder i Italien, af forsøgsudsætningerne af *Pseudomonas fluorescens* F113 *lac*ZY i 1994 og 1995.

Konklusioner

Der er ingen resultater angående effektiviteten af *P.fluorescens* F113 *lac*ZY som biologisk bekæmpelsesmiddel af damping-off, da der de to forsøgsår ikke blev observeret symptomer på sygdommen.

Innokulering med *P.fluorescens* F113 *lac*ZY havde ingen negativ effekt på nodulering på soyarødder. I 1994 var der en øget nodulering ved innokulering med både *P.fluorescens* F113 *lac*ZY og B. japonicum i forhold til de andre behandlinger. Dette blev dog ikke bekræftet i 1995.

I en rotation mellem kulturer innokuleret med forskellige GMM viser der sig ingen effekter på hverken *P.fluorescens* F113 *lac*ZY, jordens mikroorganismer eller det innoculum der blev udsat året før.

Alle de agronomiske parametre, der blev målt (spiringsprocent, noduleringskapacitet, udbytte, protein i bønner, olie i bønner, biomasse af de grønne dele, kvælstofindhold), var sammenlignelige ved behandling med *P.fluorescens* F113 *lac*ZY og ved kontroller.

P.fluorescens F113 *lac*ZY viste sig som en god kolonisator af jord og rhizosfære, med relativt forhøjede densiteter i dyrkningsperioden og derefter faldende densiteter i tiden efter. Et år efter udsætningen var densiteten 2 logaritmer mindre end ved slutningen af soyaens vækstperiode (120 dage). Ved 515 dage var densiteten under detektionsgrænsen.

Der er ikke indikationer på ændringer i mikroflora/fauna i jorden.

(I forsøget fra 1994 havde *P.fluorescens* F113 *lac*ZY næsten fuldstændig udkonkurreret den naturlige population af *P.fluorescens* efter 120 dage, hvorimod *P.fluorescens* F113 *lac*ZY i 1995 forsøget kun udgjorde 11% af den samlede *P.fluorescens* population. Dette kunne tildels skyldes et skraftigt regnskyl lige efter såning i 1995. Mængden af *P.fluorescens* F113 *lac*ZY blev ikke målt efter 120 dage i 1994, som det var tilfældet i 1995.)