Further information on the bacteria:

Responses, submitted by the "RHIZODEGRADATION" project consortium, to questions raised by Skov- & Naturstyrelsen, the competent authority of Denmark:

Response submitted on 20 March 2000

Response submitted on 24 March 2000

Response submitted on 20 July 2000

Response submitted on 1 February 2001

Response submitted on 13 March 2001

Response submitted on 20 March 2000

1. Degradation intermediates produced by the gene product of the bph genes from B. cepacia LB400:

The Bph enzymes catalyze a degradation pathway from biphenyl to acetyl-CoA. The same enzymes attack chlorinated biphenyls co-metabolically: The ring moiety with the lesser Cl-substitution (including no Cl-substitution) is thereby metabilized as above, the Cl-atoms being removed (enzymatic dechlorination under aerobic environmental conditions). The ring moiety with the higher degree of Cl-substitution is transformed to the corresponding Cl-benzoate and released by the bacterium. Reference: Bopp 1986

2. PCB degradation in the experimental soil:

In microcosm experiments with experimental soil from the field site, and with clean agricultural soil spiked with 4-chlorobiphenyl (4-PCB) we were not able to quantify chlorbenzoates formed. Apparently, these were below the detection limit of 2 mg/kg soil. Indeed, the strain *P. fluorescens* F113rifpcb in our experiments did not show high PCB degradation activity in contaminated soil. As can be seen on the graph included (Fig. 1), 4-PCB was removed by the plant relatively rapidly, the degradation effect of strain *P. fluorescens* F113rifpcb being non-significant. Considering its gene expression system (low constitutive gene expression), we actually did not expect this strain to be a good biodegrader. Within our research and development efforts, strain *P. fluorescens* F113rifpcb ought to be considered a prototype for safety studies.

3. Quantities of degradation intermediates expected, incl. their localization:

Chlorobenzoates would be expected to be formed in the root zone of the inoculated plants. In our laboratory experiments with willow, numerous chlorobenzoates were tested for their fate in a hydroponic system. The results show that chlorobenzoates are rapidly removed by willow roots. Their fate in the plants is suggested to be metabolic conversion by the plant (Chlorobenzoates are known and used as herbicides. At higher concentrations, also in our hydroponic experiments, they kill the plants. However, plant toxic concentrations are several orders of magnitude higher than what we expect to see in the experimental soil). For details, see the enclosed copy of Lagergren, 1999.
Chlorobenzoates are compounds with a short residence time in natural soil. Especially in the rhizosphere, the natural microflora is expected to readily degrade such compounds (Siciliano & Germida 1999).

4. Personal protective gear to be used:

We don't see why anything special is required beyond normal practice. We plan to protect the staff exactly the same way we do in the laboratory (lab coats, disposable gloves). In addition, we propose

to limit the possible spread of the organism via human contact, i.e., we shall use maximum care to prevent accidental contamination:
plants seeds and willow cuttings inoculated in lab and then transfered to site.
workers wear disposable protective clothing and shoe protectors during planting.
following planting this clothing is bagged, removed and autoclaved at NERI, Roskilde.

5. Biocontrol activity of P. fluorescens F113:

The biosynthetic locus, which encodes the polyketide pathway responsible for the production of 2,4-diacetylphloroglucinol (Phl), is harboured by bacteria which are present in the soil and are natural colonisers of many plant-root systems. In addition, the production of this secondary metabolic compound has been implicated in the suppression of black root rot of the tobacco plant (Keel et al., 1992; Keel et al., 1990), take-all of wheat, (Keel et al., 1992; Thomashow and Weller, 1995) Pythium damping-off of sugarbeet (Fenton et al., 1992), bacterial soft-rot of potato (Cronin et al., 1997a), and potato cyst nematodes (Cronin et al., 1997b). In a recent study by Picard et al. (2000) the frequency of occurrence of 2,4-diacetylphloroglucinol-producing pseudomonads in the maize rhizosphere has been described. Of the 1716 isolates that were recovered from the soil, 188 were capable of producing the antifungal metabolite Phl.
The amount of Phl produced by *P. fluorescens* strain F113 was reported by Fenton et al. (1992) as 0.46 ± 0.028 microM in an in vitro study when cells were cultured on minimal media agar plates (sucrose asparagine media, amended with 100 microM $FeCl_2$) for 4 days at 14 ^oC. In a similar strain CHAO of *P. fluorescens* an in planta study was conducted and the levels of Phl produced by CHAO in the wheat rhizosphere were between 0.3 to 1.2 nmoles per g of rhizosphere, the cell density which corresponds to this level of production is 8.7-8.9 $\log_{10}$ cell forming units per gram of root (Maurhoferet al., 1995).
Hydrogen cyanide (HCN) is an exclusive metabolite of some species of *Chromobacterium* and *Pseudomonas* (Leisinger & Margraff, 1979) and is formed as a degradation product of glycine. HCN production by fluorescent pseudomonads is implicated in the suppression of black root rot of tobacco by *Thielaviopsis basicola* (Voisard et al., 1989).
Voisard et al. (1994) describes that the concentrations of HCN (~100 microM) which are produced in vitro by *P. fluorescens* CHAO towards the end of the exponential growth phase are insufficient to cause growth inhibition of the CHAO strain and other fluorescent pseudomonads but inhibit the growth of the pathogenic fungi *Thielaviopsis basicola* and *Septoria tritici.* In a quantitative assay Laville et al. (1998) demonstrated that strain CHAO produces 89 ± 5 microM HCN in minimal media with oxygen limitation.

6. Confirmation of the genetic insert in P. fluorescens F113::lacZYrif and P. fluorescens F113rifpcb:

PCR experiments show presence of key *bph* genes in the organism. The vector worked as planned - stable insert - no further transposition no vector sequence i.e. no plasmid DNA present and no antibiotic resistant determinant present,. This has been reported in Dowling et al 1993 and Brazil et al 1995 (plus unpublished results).
However, we have not looked for the absence of non-required vector sequences by hybridisation or PCR. In the paper by Herero et al (1990) and DeLorenzo et al (1990) extensive analysis showed that these vectors worked as planned and no vector sequences were incorporated into the host strain.

7. Details on the selectable marker gene in P. fluorescens F113rifpcb:

This gene whose product encodes a herbicide resistant phenotype (resistance to bialaphos)
It was used as selectable marker in the lab during the construction of the strain. It is not expected to have any ecological consequences as soil organisms spontaneously produce resistant derivatives at very high frequency. This is reported in Herrero et al (1990) and De Lorenzo et al (1990).

Response submitted on 24 March 2000

1. Legal problem with Burkholderia cepacia LB400.

We regret the formal oversight with strain LB400. The strain was planned for inclusion in the field experiment as a reference treatment. As this bacterium is not genetically modified, we assumed that we were free to use it without formal permission.
If the competent authorities judge the legal situation to require a formal notification for the use of this bacterial strain within the release experiment, and if such amendment to the notification would cause a significant delay in the notification procedure, we prefer to withdraw *Burkholderia cepacia* LB400 from the experimental plans.

2. Fluorescence as an identification tool is being critisized for being quite uncertain.

We agree with this remark.
Indeed, such observations led us to switch to rifampicin resistant strains of F113. Our experimental plans are targetted to rely on the combination of rifampicin resistance with beta-galactosidase activity (strain F113::lacZYrif) and 2,3-dihydroxybiphenyl-dioxygenase activity (F113rifpcb), respectively. In both cases, the identification by growth on rifampicin plates in combination with the colour of the colonies is unequivocal.

3. F113rifpcb:

A. Is there a risk of spreading based on a selective advantage of the strain outside the contaminated area, because it may metabolise OTHER compounds by virtue of the bph-insert?

We consider this risk is minimal because:

- There is no evidence that the *bph* cassette allows the metabolism of OTHER compounds besides biphenyl and PCBs (although these genes show some homology to other pathways such as Tol and Nah and individual enzymes may use some intermediates as substrates, there are no reports in the quite extensve literature on strain LB400 (see references, part 2, in the application) showing that the *bph* operon from LB400 confers the ability to grow on substrates such as toluene or napthalene, or any other substrates.
- Metabolic profiling using BIOLOG, which reports on the metabolism of 95 different substrates, shows no differences between the parent F113 and F113rifpcb. (Sherlock and Dowling , unpublished)
- Unpublished data (Brazil, O'Gara and Dowling) from experiments looking at colonisation dynamics in the sugarbeet rhizosphere in soil amended with 4-chlorobiphenyl, indicated no difference between the parent F113 and the GMO F113rifpcb. Suggesting that even in the presence of a selection pressure (4-chlorobiphenyl) the GMO does not have a selective advantage in the rhizosphere over the parent F113. This is not surprising, and was anticipated, as these are rhizosphere strains and we predict they make use of the carbon exudates in the root niche.

B. Does the bialaphos-resistance give resistance to other selective agents, such as the herbicide Basta?

This has not been tested, however the active ingredient of Basta -- glyfosinate ammonium -- is considered the same as phosphinothricine, the active ingredient in Bialaphos.
We offer the following genetic safety considerations:

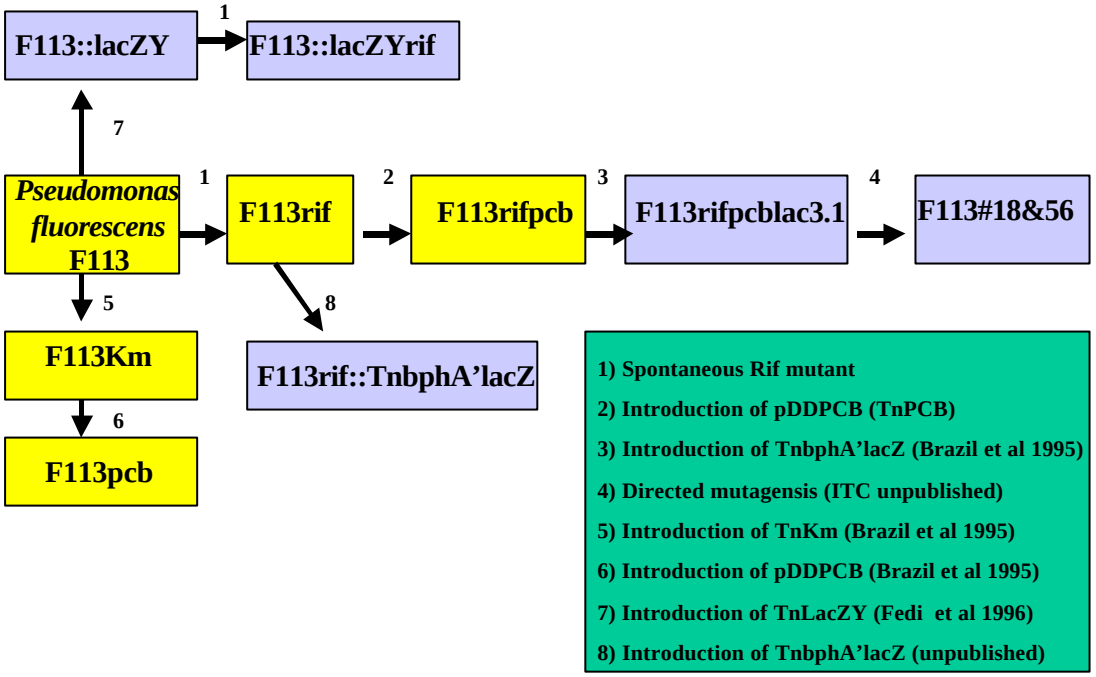
Can strain F113rifpcb grow on Basta?
The resistance gene does not confer the ability to metabolize glyfosinate. The gene product, a phosphinotricin acetylase, only results in resistance (De Lorenzo et al 1990; Herrero et al 1990). In addition, environmental concentrations of glyfosinate (see below) would be too low to support bacterial growth, even if the GMO had a metaboloc pathway for the compound.

What is the effect of a Basta application on the soil microflora?
This is a research topic, not an area of knowledge. However, we propose a model calculation: A high dose of Basta in forestry is 7.5 L/ha, in agriculture 3 L/ha. Assuming a uniform distribution of the active ingredient in 6.6 cm topsoil, the forest soil would receive 1.5 mg/kg glyfosinate on a soil basis (Arne Helweg, personal communication). If the compound is adsorbed strongly, it would be more concentrated in a thinner top soil layer, but the free concentration in soil solution, i.e., the concentration relevant for the microflora, would also be reduced drastically. Therefore, it is fair to accept the calculated concentration of 1.5 mg/kg as a maximum bacterially relevant environmental concentration after a Basta application. We propose to compare this concentration with the concentration used in the laboratory to achieve selectivity, 100 mg/kg (De Lorenzo et al 1990; Herrero et al 1990). As one can see, the concentration in the field will be roughly 2 orders of magnitude below the practical selective concentration.

How common is glyfosinate resistance in the soil microflora?
It may be worth mentioning that this ”herbicide” is naturally produced by some *Streptomyces* strains, which are.common soil microorganisms. Producing strains also carry resistance genes. Glyfosinate resistance should not be expected to be an unusual property among soil microorganisms.
More importantly, Herrero et al (1990) report practical problems with the application of the marker gene during laboratory manipulations (which, however, can be considered an environmental advantage): Spontaneous resistance to glyfosinate is high, on the order of 10^{-5}. Indeed, D. Dowling (pers. comm.) while constructing F113rifpcb, encountered spontaneous resistance mutations at a rate of approx. 90%. Therefore, in soils treated with the herbicide, a significant population of indigenous tolerant microorganisms should assure that the engineered bacterium containing the resistance marker will have little selective advantage.

4. How do the strains used in the microcosm work match the strains planned for the release?

The following Figure (from the application's executive summary) shows the relatedness of the different strains of Pseudomonas fluorescens F113:



Strain	Phenotypes										
F113	DAPG	HCN	Biocontrol								
F113rif	DAPG	HCN	Biocontrol	Rif							
F113rifpcb	DAPG	HCN	Biocontrol	Rif	2,3-OHBP	BP					
F113rifpcblac3.1	DAPG	HCN	Biocontrol	Rif	2,3-OHBP	BP	LacZ	Spc			
F113#18 &56	DAPG	HCN	Biocontrol	Rif	2,3-OHBP*	BP*	LacZ	Spc			
F113Km	DAPG	HCN	Biocontrol	Km							
F113pcb	DAPG	HCN	Biocontrol	Km	2,3-OHBP	BP					
F113::lacZY	DAPG	HCN	Biocontrol	LacZ	Growth on lactose						
F113::lacZYrif	DAPG	HCN	Biocontrol	LacZ	Growth on lactose	Rif					

The strains to be released (and the corresponding strains tested in microcosms) are:

F113::lacZYrif	(F113::lacZY)
F113rifpcb	(F113rifpcblac3.1; F113rifpcb)

In case 1), the difference is the spontaneous rif-resistance mutation in the strain used for the release.
In case 2), the partial difference is the lack of the lacZ insert in the strain used for the release.
These differences were caused by practical considerations (availability of the strain in the investigating partner's lab) at the time of the microcosm test.
Potential selective advantages due to the genetic inserts in the release strains – *lacZY* and *bph*, respectively – should have surfaced in the microcosm tests. Therefore, we consider the test strains respresentative of the release strains.

5. F113rifpcb: Can the 1 kbp fragment, derived from the vector, code for antibiotic resistance through recombination?

The non-recombinant plasmid RK2 (size 60kbp) encodes genes for the following antibiotic resistances; tetracycline, kanamycin and ampicillin.
The actual vector used in the construction (derived from RK2) is called pRK7813 (size 12 kbp) (Stanley et al 1987; Jones et al 1987) and it contains only the tetracycline resistance gene which is not located within the 1 kbp region (see map in Stanley et al 1987; Jones et al 1987).
While it is not known what the 1 kbp region codes for, it is known that none of the plasmid's 3 resistances encompass the 1 kbp region. In other words, it is unlikely that the 1 kbp region codes for an antibiotic resistance.
F113rifpcb is both tetracycline and kanamycin sensitive (like all fluorescent pseudomonads it has an intrinsic resistance to ampicillin, but is sensitive to piperacillin)

6. Groundwater hazard through metabolites produced by F113rifpcb?

A. Contamination

The site contamination consists of the following polychlorinated biphenyl (PCB) congeners:

PCB-28 (0.19 mg/kg)
PCB-52 (0.24 mg/kg)
PCB-101 (0.21 mg/kg)
PCB-118 (0.13 mg/kg)
PCB-138 (0.24 mg/kg)
PCB-153 (0.17 mg/kg)
PCB-180 (0.08 mg/kg)

B. Environmental Fate and Behavior of PCB

The environmental fate and behavior data are shown in table 1.

Table 1: Environmental Fate and Behavior Data - PCB

PCB-No.	Congener-Pattern	Solubility	Koc	Log Kow	Henry
		[µg/l]	[l/kg]		[Pa m³ mol⁻¹]
28	2,4,4´	158	41687	5.71	27.5
52	2,2´,5,5´	30	53703	5.79	48.5
101	2,2´,4,5,5´	9	42658	6.20	40.2
118	2,3´,4,4´,5	-	977237	6.40	12.5
138	2,2´,3,4,4´,5´	3	109099	6.83	15.8
153	2,2´,4,4´,5,5´	1	446684	6.80	36.1
180	2,2´,3,4,4´,5,5´	-	9415466	7.36	13.2

As one can see the most mobile congener is PCB-28 because of the relatively high water solubility and the low adsorption tendency, expressed by the Koc-value.

For that reason it is sufficent to focus the risk assessment of PCB-distribution in the environment to PCB-28.

C. Distribution of PCB in the Environment including Microbial Degradation Products

The release of PCB-28 into soil leads to the following distribution in soil compartments:
Fraction in aqueous phase: 2.8 E-4
Fraction in gaseous phase: 2.0 E-6
Fraction in soil matrix: 0.999

The velocity of PCB-28 movement in vertical direction in soil amounts to 2.76 E-7 m/d, calculated from data shown in table 1. It can be concluded that there is a neglectable vertical transfer within the soil column. Leaching to groundwater does not take place.

In strain F113rifpcb the microbial degradation intermediate from PCB-28 is 2,4-Dichlorobenzoic Acid (2,4-DCBA) (Bopp 1986). Environmental fate and behavior data from this compound are shown in table 2.

2,4-DCBA is more mobile than PCB-28, caused by the high water solubility and the very low adsorption tendency. The calculation of distribution from 2,4-DCBA in soil compartments gives the following values:

Fraction in aqueous phase:	0.999
Fraction in gaseous phase:	5.8 E-6
Fraction in soil matrix:	0.01

Using data from Table 2 the velocity of vertical 2,4-DCBA movement in soil was calculated with 9.89 E-4 m/d.

The main transfer pathway of 2,4-DCBA in the soil column is the advective transport with the soil leachate.

Table 2: 2,4-Dichlorobenzoic Acid – Environmental Fate and Behavior Data

Solubility	Koc	Log Kow	Henry
[µg/l]	[l/kg]		[Pa m^3 mol^{-1}]
251 E+3	271	2.82	2.18 E-2

D. Prediction of 2,4-DCBA Concentration in Soil

For the prediction of 2,4-DCBA concentrations in soil, caused by the degradation of PCB-28, an one-dimensional analytical soil model was used. Based on the assumption, that 10% of PCB-28 will be converted in 2,4-DCBA within 6 months, the 2,4-DCBA concentration profile was calculated, regarding realistic environmental conditions (e.g. precipitation, evapo-transpiration). Results of the calculations are shown in table 3.

Table 3: Concentration Profile of 2,4-DCBA in Soil

Depth	Conc.
[m]	[µg kg^{-1}]
0.25	4.73E+00
0.5	5.20E+00
1	1.00E+00
1.5	4.73E-02
2	8.00E-03

E. Conclusion:

In a vertical distance of 2 m from the contaminated layer (start concentration 2,4-DCBA: 0.014 mg/kg) the 2,4-DCBA concentration in soil after 360 days is estimated with 8 E-3 µg/kg located in soil water (that is 0.000008 mg/kg).
These data show that no adverse impacts to the surrounding environment (groundwater) of the site will be caused by the formation of 2,4-DBCA and its subsequent vertical movement in the soil column.

F. Qualifying Remarks:

These model calculations were performed assuming that 10% of the soil PCBs are transformed to chlorobenzoates in a given time span, but that all of the chlorobenzoates formed in that period persist in soil solution for the length of tat period. Actually, the persistence of chlorobenzoates in soil, in particular in rhizosphere soil (where they would be formed, as strain F113rifpcb will be mainly present and active in the immediate vicinity of plant roots), is extremely low due to microbial degradation and plant uptake:

- In our laboratory experiments with willow, numerous chlorobenzoates were tested for their fate in a hydroponic system. The results show that chlorobenzoates are rapidly removed by willow roots. Their fate in the plants is suggested to be metabolic conversion by the plant (Chlorobenzoates are known and used as herbicides. At higher concentrations, also in our hydroponic experiments, they kill the plants. However, plant toxic concentrations are several orders of magnitude higher than what we expect to see in the experimental soil). For details, see Lagergren, 1999, copy enclosed).
- Chlorobenzoates are compounds with a short residence time in natural soil. Especially in the rhizosphere, the natural microflora is expected to readily degrade such compounds (Siciliano & Germida 1999). Indeed, chlorobenzoates are not on the pollutant list of any of the European country's EPA. Although chlorobenzoates are compounds used for various industrial and agricultural applications, involving intentional and unintentional spills, there is no report of a chlorobenzoate-contaminated site or aquifer. Knowing about their rapid degradation in soil explains why this is so.

Response submitted on 20 July 2000

1. How does the bph operon function in F113rifpcb, as compared with LB400? (Is gene expression at the same level, induced with the same efficiency, as in the donor?)

Expression as measured by enzyme activity and growth on biphenyl is thought to be similar to LB400. In LB400 the pathway is considered to be weakly inducible (X2-3 fold) (Erickson & Mondello 1992, J. Bacteriol., May 1992, 2903-2912, Vol 174, No. 9) and this seems to be the case in F113rifpcb. All the data concerning the F113 strains are published in Brazil et al 1995 (Specifically see fig 1).

2. What are the PCB-removal rates and the degradation products in the experimental soil, as affected by F113rifpcb?

Presently there are no data re. the experimental soil.

3. Is the biocontrol activity of F113::lazZYrif and F113rifpcb similar to the wildtype?

F113::lacZY:
A bioassay known as The Bacillus test was employed for this purpose (Fenton et al., 1992 Appl Environ Miccrobiol 58, 3873-3878). This consists of spraying a suspension of Bacillus subtilis culture on an agar plate with F113lacZY and F113 wildtype.
Both F113 strains were grown to the same optical density, centrifuged and washed in buffer. Fifity microliters were then spotted on the plates and they were left to grow overnight. The plates were then sprayed with Bacillus subtilis.
After 10, 20 , 30 hours the diameter of the halo derived from the production of 2,4-diacetylphoroglucinol of the Pseudomonas strains were monitored. F113 amd F113LacZy showed the same diameter of the halo at the same time points (1.5- 2 cm).

F113rifpcb:
Unpublished work from the lab of D. Dowling, ITCarlow and from Danisco Seed (confidential data submitted separately), and also Brazil et al (1995) show that the key traits of F113 responsible for biocontrol -- HCN, DAPG, siderophore, protease, and colonisation -- are present in F113rifpcb. In addition work in our lab (unpublished) shows that F113rifpcb is antagonistic to the fungal pathogen *Pythium ultimum* at similar levels (in an in vitro) assay as the WT strain. However, we have not done biocontrol tests in soil.

4. There also was a question regarding risk assessment of PCB metabolites.

This was already answered by a model calculation provided in an earlier letter.

5. Construction of F113rifpcb and F113lacZY:

We were asked to provide a step-by-step description of the construction process, including original data (gels, ...) for verification of the construction steps, PCR results, etc..

F113::lacZYrif:

There were five F113LacZY mutants randomly chosen. Genomic DNA of each one was digested with the restriction enzyme SalI overnight and then transferred on nylon membrane.
The probe was the lacZY fragment which was digested with NotI and purified on low melting agarose and labelled with S35dA. SalI cut the lacZY in the middle, resulting in 2 two visible bands of hybridisation in the appropriate lanes of a Southern blot (submitted separately).

F113rifpcb:

The three papers, Brazil et 1995, Dowling et al 1993 and Fedi et al 1996, adequately describe the construction of the strains. The inclusion of original gels etc. is impossible as most of this work is up to 10 years old and will be difficult to track down. Additional experiments were carried out (see pages 17-18, 23 and these confirm the construction process).

Two particular points also ought to be covered in detail: Identity and function of the „unknown genes“ in F113rifpcb, and function of the Basta-resistance in F113rifpcb:

- Orf O is a possible regulatory gene of bph
- Bar (Basta resistance) was used in the original vector construction by De Lorenzo et al and Herrero et al (copies of these papers provided), and it was used in the selection process in the construction of pDDPCB (Dowling et al 1993) in *E.coli*, the intermediate vector in the construction of F113rifpcb. As we pointed out it is not a particularly useful selectable marker in *Pseudomonas* as the F113 spontaneous mutation rate to Basta resistance is high.
- The artifact of the cloning procedure ~1kbp, is from the vector pRK7814, it does not contain functional antibiotic resistance genes. (preliminary data based on partial DNA sequencing indicate it contains a truncated lac Z gene, from the pRK cloning site). Please note that this fragment is in fact ~650bp in size.

More information on regions of “unknown” function:

1) OrfO - function is not known experimentally but is thought to be involved in *bph* operon regulation due to homology with other DNA regulatory proteins.

BLAST analysis shows homology with GNTR family of transcriptional activators…

: P37335 . HYPOTHETICAL TRANS...[gi:586491] PubMed, Related Sequences

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ACCESSION   P37335
PID         g586491
VERSION     P37335  GI:586491
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DBSOURCE    swissprot: locus YBPA_BURCE, accession P37335;
            class: standard.
            created: Oct 1, 1994.
            sequence updated: Oct 1, 1994.
            annotation updated: Nov 1, 1997.
            xrefs: gi: 349602, gi: 151083, gi: 285357
            xrefs (non-sequence databases): PFAM PF00392, PROSITE PS00043
KEYWORDS    Hypothetical protein; Transcription regulation; DNA-binding.
SOURCE      Burkholderia cepacia.
  ORGANISM  Burkholderia cepacia
            Bacteria; Proteobacteria; beta subdivision; Burkholderia group;
            Burkholderia; Burkholderia cepacia complex.
REFERENCE   1  (residues 1 to 245)
  AUTHORS   Erickson,B.D. and Mondello,F.J.
  TITLE     Nucleotide sequencing and transcriptional mapping of the genes
            encoding biphenyl dioxygenase, a multicomponent
            polychlorinated-biphenyl-degrading enzyme in Pseudomonas strain
            LB400
  JOURNAL   J. Bacteriol. 174 (9), 2903-2912 (1992)
  MEDLINE   92234948
  REMARK    SEQUENCE FROM N.A.
            STRAIN=LB400
COMMENT     ---------------------------------------------------------------
--
            This SWISS-PROT entry is copyright. It is produced through a
            collaboration between the Swiss Institute of Bioinformatics and
            the EMBL outstation - the European Bioinformatics Institute.
            The original entry is available from http://www.expasy.ch/sprot
            and http://www.ebi.ac.uk/sprot
            ---------------------------------------------------------------
-.
            [SIMILARITY] BELONGS TO THE GNTR FAMILY OF TRANSCRIPTIONAL
            REGULATORS. STRONG, TO E.COLI YGAE.
FEATURES             Location/Qualifiers
     source          1..245
                     /organism="Burkholderia cepacia"
                     /db_xref="taxon:292"
     Protein         1..245
                     /product="HYPOTHETICAL TRANSCRIPTIONAL REGULATOR IN BPHA
                     5'REGION"
     Site            56..75
                     /site_type="dna-binding"
                     /note="H-T-H MOTIF (BY SIMILARITY)."
ORIGIN
        1 mnartpnslt mggdksfada spvpianvrs lieatfqrlr adivegrlaa gsrlaiedlk
       61 sryevsggtv realsllvan nlvqtqaqrg fhvtpmsldd mrdlaatria lecealrqsv
      121 lngdaewear vvssyhrlsl ldertmrdpv hlfnqweqan rdfhealisa cssawtqrfl
      181 silylqmery rrltamhnrp arnvheehla lrdsalarda erctellrmh iessisvvrq
      241 fgllr
```

Conclusion: BLAST analysis shows no homology with genes implicated in pathogenicity.

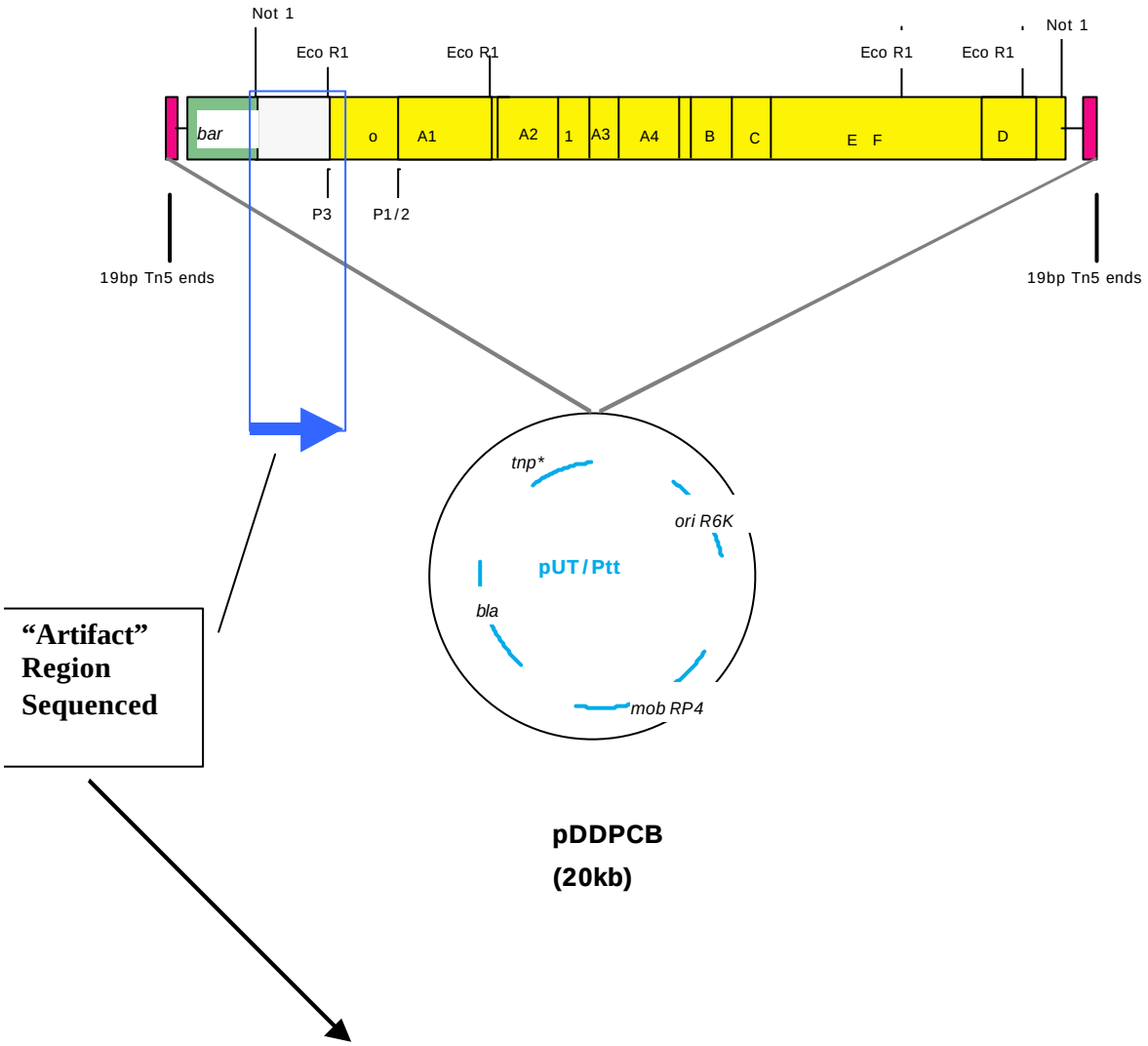
2) ~1kbp fragment from broad host range plasmid RK2 (an artefact of the intermediate cloning process) has no known phenotype.

The cloning vector used in this construction (see Dowling et al 1993) was pRK7813, a 11.5kbp cosmid cloning vector derivative of RK2 (60kbp). The construction of pRK7813 is described by Jones and Gutterson (1987) Gene 61:299-306 and it contains the pUC9 lacZ alpha fragment and multiple cloning site.
pRK7813 was used to make a (ecoR1) gene library of LB400 (Dowling et al 1993) and 2 overlapping cosmid clones were isolated. These were recombined in vivo to produce a cosmid clone pDD530 with the complete pathway, which was subsequently used to construct pDDPCB (see Dowling et al 1993 for details).
In that construction process use was made of a Not1 site on the vector pRK7813 located near the MCS (~700bp) to excise the bph region as a single Not 1 fragment for cloning in pUTPttr (de Lorenzo et al 1990) – this led to the incorporation of a small amount of pRK7813 vector sequence in the final construct (pDDPCB).
A subcloning experiment was carried out of pDDE1 (a subclone of pDD530) containing most of the bph pathway) by cutting with Not1 and Sal1 with a view to cloning this pRK7813 region into pGEM T for DNA sequencing.
DNA sequence (~750bp) from the Not1 site is now available and BLAST analysis shows ~300bp identity with a non-coding region of pUC9 and ~148bp have identity with the a subunit of beta-galactosidase (- the N terminal region) .
There is no homology to the beta-lactamase gene or any other antibiotic resistance genes.

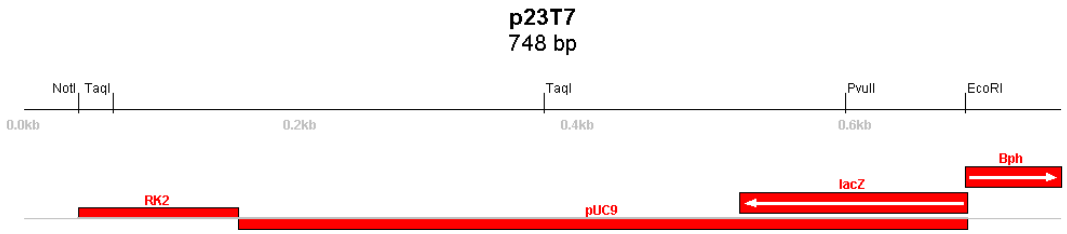
A functional map of the sequenced region is shown in Fig 1a-b. Red boxes in Fig. 1b show complete identity with the named sequence. None of the DNA sequence contains antibiotic resistant genes. The pUC9 region is common to all pBR322 based vectors. It appears to be located in a non–coding region of the vector. Translation of this region in all possible reading frames and BLAST analysis indicates no significant homology to antibiotic resistant determinants.

In conclusion this data is in agreement with published maps in Dowling et al 1993 and confirms that this region is derived from the original vector pRK7813 and it encodes no functional genes. Finally, the DNA sequence of the entire PCB cassette located within F113rifpcb is now known.
(The PCB cassette, as defined in figure 1a, contains the *bph* operon , bar gene and pRK7813 vector sequences. The entire bph operon has been sequenced and this information was included in the original application. Likewise the bar gene and now finally the pRK vector sequence is available.)

Fig 1 a) Location of pRK7813 “artifact” in pDDPCB



1 b) Summary of DNA sequence analysis: identity of pRK “artifact” sequences



Response submitted on 1 February 2001

1. How does the bph operon function in F113rifpcb, as compared with LB400? (Is gene expression at the same level, induced with the same efficiency, as in the donor?)

Expression as measured by enzyme activity and growth on biphenyl has been shown to be similar to LB400. In LB400 the pathway is considered to be weakly inducible (2-3 fold) (Erickson & Mondello 1992, J. Bacteriol., May 1992, 2903-2912, Vol 174, No. 9) and this seems to be the case in F113rifpcb. All the data concerning the F113 strains are published in Brazil et al 1995 (copy enclosed, specifically see Fig 1): BphC enzyme activities were compared between LB400 and F113rifpcb (Fig 1 Brazil et al 1995) in the presence of succinate and Biphenyl. The results show a broadly similar pattern of activity between the two strains. Furthermore, in the same Figure 1 in Brazil et al (1995) growth curves on BP and succinate were presented, again there seems to be no major difference in growth rates on these substrates.
Further information is provided on page 1948 of Brazil et al (1995), "Regulation of *bph* operon in F113pcb.".
The major differences between F113pcb and LB400 are genetic stability (F113 excellent - LB400 poor), rhizosphere colonisation (F113 excellent -LB400 fair), and of course F113 is not a *Burkholderia* sp.

2. Construction of F113rifpcb:
We were asked to provide more evidence for verification of the DNA inserted.

A. Construction:

The construction of the delivery vector (Herrero et al 1990, and De Lorenzo et al 1990), the PCB cassette (Dowling et al 1993) and the GMO strain F113rifpcb (Brazil et al 1995) have been described.

We presented separately unpublished (therefore confidential) evidence to confirm the construction process. A southern hybridisation experiment was done with F113rifpcb and other *bph* containing strains. The *bphC* gene was used as a hybridisation probe. The data indicate that pDDPCB works as expected i.e. introduces random insertions into the target genome and in the case of F113rifpcb the *bph* cassette is present as a single insertion on a ~ 20 Kb HindIII fragment of chromosomal DNA.

B. Presence or absence of delivery vector sequences:

In our view the relevance of this question to any risk assessment concerns the issue, whether there is phenotypic expression of the following two vector genes in the newly constructed GMO;
1) Vector selectable marker Bla (β-lactamase)
2) Vector Tnp (Tn5 transposase).

Absence was confirmed by two experiments to detect the phenotypic expression of genes carried by the delivery vector.

1. beta-lactamase gene *bla*. In *Pseudomonas* this gene confers resistance to the antibiotic piperacillin. F113rifpcb was tested for resistance to this antibiotic and compared to wildtype (WT) F113 and other strains harbouring the PCB cassette. F113rifpcb was found to be sensitive to piperacillin similar to the WT, indicating the absence of a functional *bla* gene (unpublished, hence confidential data submitted separately).

2. Transposase gene *tnp*. The transposase gene is carried by the delivery vector and is required to integrate the cassette into the target chromosome and subsequently is lost due to non-replication of the vector (Herrero et al 1990 and De Lorenzo et al 1990). Monitoring the re-transposition of the cassette to a target plasmid (RK2) tested the functional absence of this gene. Whereas the transposon Tn5 could transpose from the genome of a F113rifpcb derivative at a frequency of 10^{-5} per recipient, the transposition of the *bph* cassette was undetectable $> 10^{-9}$ per recipient. Therefore we conclude the absence of a functional transposase *tnp* gene (relevant data submitted separately).

3. Does P. fluorescens F113 produce mupirocin?

For background information on mupirocin (pseudomonic acid) please refer to the monograph appended. The information available suggests that bacterial production of the antibiotic is limited to fermentation culture, and to specialized strains. Nevertheless, we did investigate whether *P. fluorescens* F113 has the capability to produce mupirocin. In view of the challenges of setting up a specialized fermenter culture and running the appropriate analyses, we arranged with SmithKline Beecham to have the wildtype strain of *P. fluorescens* F113 tested for mupirocin production at their fermentation laboratory in England. SmithKline Beecham is the only manufacturer of mupirocin in the world. It was established by SmithKline Beecham that *P. fluorescens* F113 does not produce mupirocin. The following email from Dr Barry Barton, Biotechnology Development Unit , SmithKline Beecham, confirms this finding.

>From: Barry.A.Barton@sb.com
>Date: Tue, 28 Nov 2000 16:37:23 +0000
>To: j.morrissey@ucc.ie
>Subject: F113 and mupirocin
>
>Dear John,
>this is just a quick note to say that we have finally found some time to
>ferment this strain, and from our hplc analysis we have concluded that F113 does not
>make pseudomonic acid A (our detection limit is 1ug/ml).
>We set up multiple fermentations in this experiment, but did not make a repeat.
>Regards
>Barry

4. Is P. fluorescens F113 a potential human pathogen?

Pseudomonas fluorescens is an ubiquitous bacterium that can be isolated from most soils, frequently associated with plants roots. The species possesses a potential biotechnological use and is included as a live organism in comercial formulations intended for biocontrol (BlighBan A506; Conquer). The strain F113lacZ has been deliberately released in experiments performed in several European countries in the frame of the EU funded IMPACT programm.
However, there are several reports in the medical literature linking *P. fluorescens* strains with human and animal illness, although the lack of precise species identification in some of these reports leaves it unclear, if the isolates are true *P. fluorescens* strains or fluorescent pseudomonads (a group that includes other species such as *P. aeruginosa* and *P. putida*). In more detail, on 30 January 2001 a search in the Medline database, using the terms "Pseudomonas fluorescens" and "infection", yielded 60 records (reproduced in the appendix).
However, 34 of these records turned out to be irrelevant for pathogenesis of *Pseudomonas fluorescens* because they fall in either of these cathegories:

- They refer to plant pathogenesis
- They refer to infection by fluorescent pseudomonads (not *Pseudomonas fluorescens*).
- They report experiments showing general characteristics of *P. fluorescens*, such as antibiotic resistance and growth on different media.
- They are reviews showing the frequent isolation of fluorescent pseudomonads in humans and animals.

From the remaining 26 papers, 13 are also irrelevant for human health: The most frequent cause of citation is pseudobacteremia (9 papers). That is: the bacterium is isolated from the blood of the patient, but it is shown in the paper that the bacterium is not the cause of the illness. There is 1 paper showing the isolation of *P. fluorescens* from a catheter but no infection was reported. 1 paper reports the presence of *P. fluorescens* on animal bite wounds without reporting infection. 1 paper report the isolation (but not pathogenesis) of *P. fluorescens* in turkeys infected with Bordetella and Chlamydia. 1 paper shows the isolation of *P. fluorescens* from diseased rainbow trout, but it was not shown that *P. fluorescens* had caused the disease.
From the remaining other 13 citations, 1 paper reports 4 cases of bacteremia from hospitalized patients (Hsueh et al, 1998; nr. 11 on the Medline search result enclosed), the infection being related to the use of an infected catheter. All patients recovered, two without treatment and the other after antibiotic treatment. 1 paper (Zervos and Nelson 1998; nr. 13) reports the isolation of *P. fluorescens* in one out of 39 patients with a nosocomial pneumonia. No proof of the isolate causing the disease is presented. Another paper (Yamamoto et al. 1991; nr. 28) shows that out of 54 lung cancer patients that developed respiratory infections, in two patients *P. fluorescens* was isolated and considered the causative agent. The patients recovered from infection after antibiotic treatment.
Two papers (Rais-Bahrami, 1990, nr. 30, and Bompard et al. 1988, nr. 34) report neonatal septicaemia. In the first paper the authors refer to infection by pseudomonads, without the identification of the causative agent. In the second paper, *P. fluorescens* was isolated from 1 out of 7 neonates with septicaemia. The patient recovered after antibiotic treatment. Two papers deal with osteomyelitis (Dubey et al 1988, nr 36, and Hessen et al. 1987, nr. 38). In the first one, in 1 out of 24 patients, *P. fluorescens* was isolated from bone culture. No relation of the isolate with the illness was demonstrated. Furthermore, 25 bacterial isolates were obtained from 15 patients, showing that not all the isolates were the pathogenic agents. In the second paper, *P. fluorescens* was isolated from 1 out of 23 patients with osteomyelitis. No proof of pathogenicity of the isolate is presented.
Murray et al. (1987, nr. 37) indicate that *Pseudomonas fluorescens* transfusion-related septicaemia (TRS) is rare. They present the first description in the UK of two cases of TRS caused by this

organism. It is not clear how the identification was performed. Thangkhiew (1986, nr. 41) reports the successful treatment with ceftazidime of a *Pseudomonas fluorescens* chest infection in a myasthenic patient. This is an old paper and identification of the isolate is uncertain. The remaining four papers are also old papers and the precise identification of the isolates is not granted. In 1986, Shenderov (nr. 42) reported the problematic identification in these days of *P. fluorescens*. Gibaud et al. (1984, nr. 47) and Khabbaz et al. (1984, nr. 48) reported bacteremia caused by contaminated blood. Redding and McWalter (1980, nr. 54) reported *Pseudomonas fluorescens* cross-infection due to contaminated humidifier water. Finally, Sarubbi et al. (1978, nr. 57) showed a case of nosocomial meningitis and bacteremia due to contaminated amphotericin B that contained *Enterobacter agglomerans*, *Pseudomonas fluorescens*, and *P. aeruginosa*.

It is important to note that:

a) No serious cases of illness that have been undoubtelly related to Pseudomonas fluorescens have been reported in the 90s, when identification of P. fluorescens is reliable.
b) Most of the cases reported correspond to hospital patients and are directly linked with the use of contaminated products or hospital devices. There is not a single case associated with environmental acquisition of the bacterium.
c) Most of the cases did not require treatment, and the remaining few of them recovered upon antibiotic therapy.

Requests to Statens Veterinær & Serumsinstituttet and to Århus Amtssygehus, for clinical *P. fluorescens* isolates did not result in biological materials, because both institutions, although they initially believed they had such strains in their collection, later returned with the information that the strains they thought they had were not *P. fluorescens* or did not grow upon being re-checked.

Several environmental isolates of *P. fluorescens* were located in NERI's culture collection, which is being maintained at –80 C. These environmental isolates (Table 2) were tested for their temperature sensitivity by doing traditional agar plate counts at different temperature conditions. The criterium for temperature sensitivity was, therefore, colony formation at different temperatures. Results are presented in Table 2.

Plate counts were performed by plating triplicate serial dilutions on SA (sucrose-asparagine, no antibiotic) medium, which is the preferred medium for growing *P. fluorescens* in the laboratories of the consortium of release applicants. Plates were incubated at 30, 37 and 40 C (for specifics please refer to the footnote at the bottom of the Table 2). 42 C was not applied. The plate count results at 30 C were set at 1, cfu from the other temperatures were expressed as ratios thereto.

The results indicate that

- all tested strains of *P. fluorescens* were sensitive to temperatures above 30 C, as their cfu at elevated temperatures ranged from 1/10 to $1/10^6$ of the value obtained at 30 C.
- none of the isolates, with the exception of DF57 and R2F, are suited for growth at body temperature.

It should be noted that preparation of the plates involved handling at room temperature for 2–3 hours and repeated removal from the incubators for counting (2–3 hours), i.e., a total of 4–6 hours' incubation at room temperature. The cfu at elevated temperatures, therefore, is an overestimate. The measured sensitivity contains a safety margin.

Based on these results, one might question whether the strains DF57 and R2F would be suitable for environmental application without further testing. In our hands at NERI, we observed (data not shown) that repeated cultivation at 30 C increased the strains' capability of growing at 37 C. The history of our environmental isolates is not documented in terms of how many generations at 30 C are represented in our currently frozen cultures. There is a chance for the environmental isolates of having been exposed to 30 C for a substantially higher number of generations.

Our laboratory data (Table 1) show that F113 and derivatives are unable to grow at 37ºC, making it unlikely to act as a human pathogen. Only the blood injection or aspiration of huge amounts of the bacterium could cause bacteremia or respiratory diseases. The proposed use of F113, coating seeds with a solid bacterial carrier, makes these infection routes extremely unlikely.

In summary, the lack of growth at body temperature, the previous experience in deliberate release, including approved commercial applications, and the proposed release method (as coated seeds) makes it extremely unlikely that the release of strain F113 derivatives can cause any health problem. Therefore the proposed release should be regarded as safe.

5. Is the rifampicin tolerance of the release strains of regulatory concern?

The *Pseudomonas fluorescens* F113 derivatives that are planned to be released are tolerant to rifampicin, and this tolerance, together with other traits, such as β-galactosidase production, can be used to monitor the fate of the released strains. It should be noted that rifampicin tolerance is based on a spontaneous mutation in a gene encoding one of the subunits of the RNA polymerase (rendering the RNA polymerase tolerant to rifampicin), and not on a "rifampicin resistance" gene. For this reason we correctly call it tolerance, instead of resistance.
There is not a distinct gene product confering rifampicin tolerance, and not a separate genetic trait that is transferable in the same fashion as resistances to other antibiotics are transferable. Like for any other gene, mutations in the RNA polymerase subunit gene occur spontaneously. Mutations that confer rifampicin tolerance arise spontaneously at a frequency of approximately 10^{-8}. Due to the nature of the rifampicin tolerance genotype (a point mutation in the chromosome) and to the lack of selective pressure for this trait in the soil or rhizosphere, the probability of an indigenous microbe becoming rifampicin tolerant through lateral gene transfer and persistence of rifampicin tolerance is well below the probability of rifampicin tolerance arising due to spontaneous mutation.

In conclusion, because
- there is not a "rifampicin resistance gene" of regulatory concern, and
- gene transfer is less likely than spontaneous mutation,

rifampicin tolerance in F113 release strains should be regarded as safe.

Table 1: Temperature sensitivity of *Pseudomonas fluorescens* strains

determined by plate counts on SA agar: log of cfu at the temperature indicated, relative to log cfu at 30C. For example, by increasing the temperature from 30 to 37 C, the bacterial count drops by a factor of 10^6 inthe type strain ATCC 13525^T.

Name or source	30C	37C	37>30C	40>30C
Type strain (isolated from pre-filter tanks)				
ATCC 13525^T	0	-6	<-6	-4
Environmental isolates:				
F113 wt	0	-2	-1	-3
F113rifpcb	0	-3	-4	-3
F113lacZY	0	-3	-2	-3
DF57	0	-1	-2	-3
R2F [1)]	0	-1	-1	-1
DR54	0	<-6	-5	-5

Notes: All strains grew well on Gould's medium, except for strain R2F.
30C means 5 d at 30C; 37C, 3 d at 37C; 37>30C, 3 d at 37C, then 5 d at 30C; 40>30C, 1 d at 40C, then 5 d at 30C.

[1)] Van Elsas, J.D., Trevors, J.R. and Stardub, M.E. (1988) Bacterial conjugation betw. pseudomonads in the rhizosphere of wheat. FEMS Microbiol. Ecol. 53,299-306.

Response submitted on 13 March 2001

Experiment to determine the presence or absence of the bla gene in F113 derivatives
(Unpublished, hence confidential data submitted separately)

Conclusion.

These data are consistent with the phenotypic data i.e. Piperacillin (Pip) resistance/sensitivity.
The strain chosen for release (#615) is Pip sensitive, however, based on amplification with primer set B and subsequent Pst1 digestion, it contains part of the *bla* gene. But based on the inability of primer set A to amplify a ~200 bp amplicon, it lacks a 5' portion of the gene and is genotypically and phenotypically inactive.
In contrast, strains that are Pip resistant e.g. F113#12 and the positive control *E.coli* pDDpcb, amplify an additional ~200bp amplicon corresponding the N-terminal portion of the Bla protein and are functionally active.