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Genetically modified Pseudomonas biosensing biodegraders to detect PCB and chlorobenzoate bioavailability and biodegradation in contaminated soils

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Abbreviations: RT-PCR, quantitative real time-PCR

Whole cell microbial biosensors offer excellent possibilities for assaying the complex nature of the bioavailable and bioaccessible fraction of pollutants in contaminated soils, which currently cannot be easily addressed. This paper describes the application and evaluation of three microbial biosensor strains designed to detect the bioavailability and biodegradation of PCBs (and end-products) in contaminated soils and sediments. Polychlorinated biphenyls (PCBs) are considered to be one of the most wide spread, hazardous and persistent pollutants. Herein we describe that there was a positive correlation between the PCB levels within the samples and the percentage of biosensor cells that were expressing their reporter gene; *gfp*. Immobilisation of the biosensors in calcium alginate beads allowed easy and accurate detection of the biosensor strains in contaminated soil and sludge samples. The biosensors also showed that PCB degradation activity was occurring at a much greater level in Pea inoculated planted soil compared to inoculated unplanted soil indicating rhizoremediation (the removal of pollutants by plant root associated microbes) shows considerable promise as a solution for removing organic xenobiotics from the environment.

Introduction

PCBs are a group of chlorinated aromatic compounds, which are among the most widely identified environmental contaminants persisting in the biosphere.[1] Despite the abundance of naturally occurring PCB degrading bacteria, few indigenous PCB degraders with proven degradation efficacy have been described to date.[2] To address this, a number of genetically modified (GM) micro organisms have been generated with enhanced biodegradation ability.[3]

Pseudomonas fluorescens F113 was originally isolated from the rhizosphere of sugar beet and was found to be an excellent root colonizer of other plants including tomato, pea, alfalfa and willow.[4-8] It was also shown to possess biocontrol traits[9] and was chosen for gene modification for the degradation of PCBs. *Burkholderia xenovorans* LB400 is an efficient PCB degrader, however its reported poor survivability in soil has limited its use in bioremediation. In addition the PCB degradation trait is genetically unstable as it is flanked by insertion elements and repeat sequences. In order to address these problems the genes responsible for PCB degradation from *B. xenovorans* LB400, termed the *bph* operon,[10] were chromosomally inserted into a spontaneous rifampicin-resistant mutant of F113 creating *P. fluorescens* F113rifpcb.[11] Subsequently the *bph* operon was modified by cloning a strongly constitutive (nod box) promoter from *Sinorhizobium meliloti* upstream of the *bph* operon and the cassette was subsequently chromosomally integrated into *P. fluorescens* F113riflacZY, creating *P. fluorescens* F113L::1180, a more efficient PCB degrader[12] with constitutive expression of *bph.*

Biosensors are useful tools when carrying out environmental risk assessments on polluted sites or for monitoring the efficacy of a remediation strategy, due to their ability to detect only the biologically relevant (bio-available) fraction of the contaminant.[13] Liu et al. constructed three biosensor strains; *P. fluorescens* F113rif*gfp*, *P. fluorescens* F113rifPCB*gfp* and *P. fluorescens* F113L::1180*gfp* designed to detect the bio-availability and biodegradation of PCBs.[14] These strains were constructed by the chromosomal insertion of a promoter reporter construct, *xylS*Pm::*gfp*mut3 (encoded by plasmid pJBA26,[15]) into *P. fluorescens* F113rif (a non PCB degrader), *P. fluorescens* F113rifPCB and *P. fluorescens* F113L::1180 to create *P. fluorescens* F113rif*gfp, P. fluorescens* F113rifPCB*gfp* and *P. fluorescens* F113L::1180*gfp*, respectively (**Fig. 1**). The p_m promoter in this construct is derived from the TOL plasmid and regulates the

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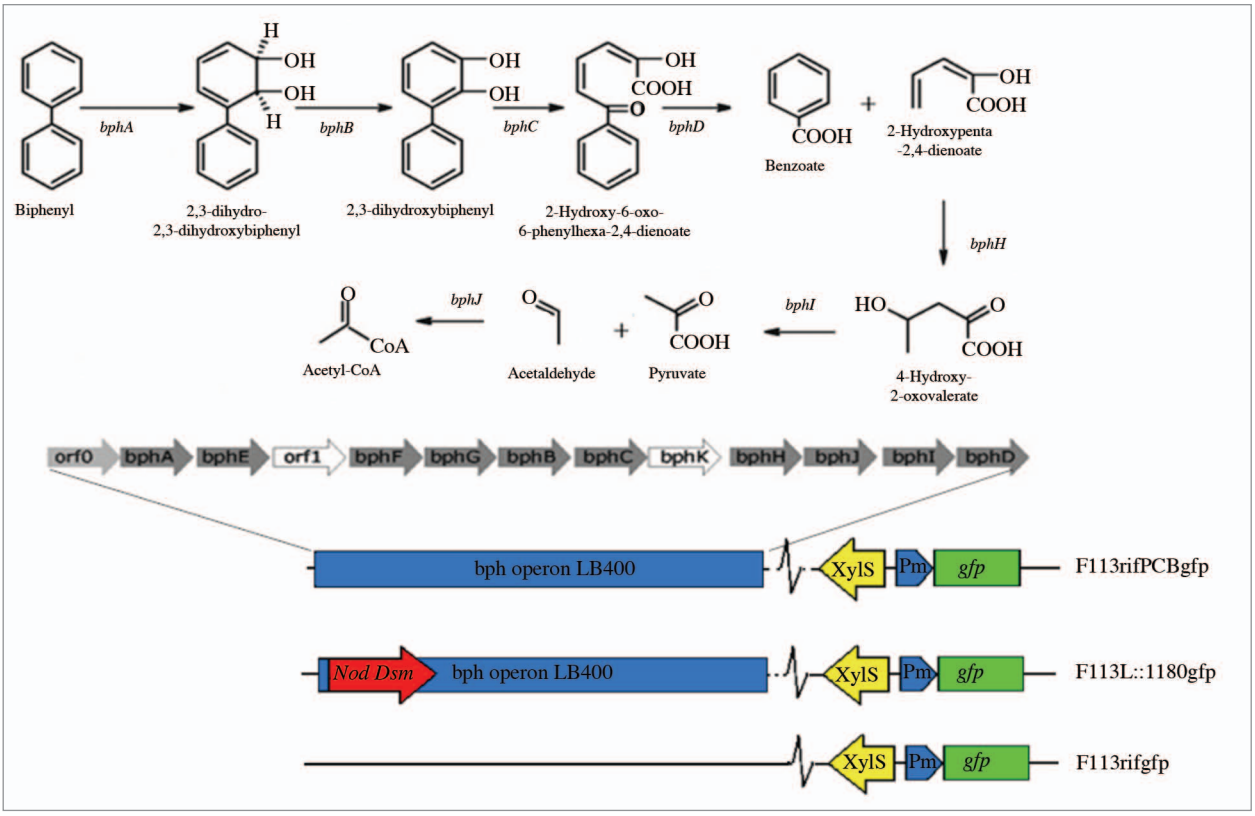

Figure 1. PCB catabolic pathway encoded by the *Burkholderia cepacia* LB400 *bph* operon and the genetic maps of the *Pseudomonas fluorescens* F113 biosensor constructs.

meta-pathway of aromatic hydrocarbon degradation. The meta-pathway is induced by chloro-benzoic acid derivatives (which are also end products of the PCB degradation pathway). Induction of the p_m promoter in the *xylS*Pm::*gfp*mut3 construct drives the expression of the green fluorescent protein (*gfp*) which is detectable using fluorescent spectroscopy or epi-fluorescent microscopy. The level of *gfp* expressed was shown to have a linear relationship with chlorobenzoate concentration. As the F113rifPCB*gfp* and F113L::1180*gfp* strains degraded PCBs they produced chlorinated benzoate end products which in turn induced the expression of *gfp*. Therefore these strains could actively report on their own degradation of PCBs.[14]

This paper describes the application of these biosensor strains to monitor PCB biodegradation in different PCB contaminated soils and sediments. It also describes their use to monitor PCB degradation activity in two different PCB remediation strategies; soil bioremediation and plant rhizoremediation.

Results

Construction of PCB biosensor strains. The suicide plasmid pJBA26 was used to introduce the reporter element by bacterial conjugation and subsequent transposition into PCB degrading engineered and WT *P. fluorescens* F113rif. Transconjugant colonies showing rifampicin (Rif) and kanamycin (Kan) resistance were purified by repeated streaking on selective plates. One transconjugant from each conjugation that visually exhibited green fluorescence by epi-fluorescent microscopy was selected and designated *P. fluorescens* F113rif*gfp*, F113PCB*gfp* and F113L::1180*gfp*, respectively. Preliminary characterisation of these strains is described in Liu et al.[14] which confirmed the presence of the *gfp* gene and showed that there was only one insertion event in each of the transconjugants. The specific growth rate and phenotypic metabolic profiles of the transconjugants were identical to those of their respective parent strains. This suggests that the chromosomal insertion of the *gfp* construct did not affect any major catabolic pathways within the transconjugant strains. A graphical representation of the genetic modifications and the encoded *bph*/PCB metabolic pathway encoded by these GM strains is shown in **Figure 1**. In vitro tests showed that the biosensors were induced by 3-chlorobenzoate (3-CBA), 2,3-dichlorobenzoate (2,3-DiCBA) and 3,5-dichlorobenzoate (3,5-DiCBA) with a linear relationship (ranging from 4 to 400 ppm) between the fluorescent intensity and the $\log^{10}$ concentration of the inducer. The sensitivity of these biosensors towards 3-CBA, 2,3-DiCBA acid and 3,5-diCBA acid was found to be 0.1 ppm using epi-fluorescent microscopy. The induction of the *gfp* expression started after 6 h and the final fluorescence intensity reached a maximum at the end of exponential growth, and was stable in the stationary phase after 14 h. 2-chlorobenzoate (2-CBA), 4-chlorobenzoate (4-CBA) and 3,4-dichlorobenzoate (3,4-DiCBA) did not result in any significant induction of *gfp* expression. The PCB congeners 3-chlorobiphenyl (3-CBP), 2,3-dichlorobiphenyl (2,3-DiCBP) and 3,5-dichlorobiphenyl

(3,5-DiCBP) induced *gfp* expression in the PCB degrading biosensor strains (F113PCB*gfp* and F113L:1180*gfp*) but not in F113rif*gfp* (non-PCB degrader). These results indicated that biosensors F113PCB*gfp* and F113L::1180*gfp* could report their own PCB degrading activity.

Evaluation of alginate bead encapsulated biosensors in PCB contaminated environmental samples. To test the detection ability of the biosensors in situ, alginate encapsulated biosensors *P. fluorescens* F113rif*gfp* or *P. fluorescens* F113L::1180*gfp* were introduced to PCB contaminated soils/sediments from different geographical sites. PCB congeners were analyzed in these samples and the results are shown in **Table 1**.

Fourteen days after the introduction of the encapsulated biosensor F113L::1180*gfp* into the PCB contaminated soil and sludge samples, 8–35% of the total number of bacterial cells within the beads were expressing *gfp* (**Table 2**). There was a positive correlation between the numbers of *gfp* expressing cells and increased PCB contamination within the samples and this followed the order A>B>C>D (**Fig. 2**). In the D soil sample (uncontaminated soil) just 0.01% of bacterial cells within the bead were fluorescent. This was considered as a background fluorescent fraction of the strain and has been observed for other biosensor strains.[15]

After introducing the encapsulated F113rif*gfp* biosensor into the PCB contaminated soil and sludge microcosm samples less than 0.01% of the total number of bacterial cells within the beads were expressing *gfp*. The same level of fluorescent cells was observed when this biosensor was introduced into the D sample soil (uncontaminated soil) 14 d after inoculation.

Soil survivability and plant colonization ability is improved by encapsulation in alginate beads. In unplanted soil that was treated using direct inoculation with the biosensors, the average initial population of the two biosensor strains was 1 x 10^8 CFU g^{-1} and after 1 month the average population was 1 x 10^4 CFU g^{-1} soil or less. This represents just 0.01% of the inoculum remaining after 1 month. There was no significant difference in the population sizes of the two PCB biosensor strains in the unplanted soil (ANOVA p = 0.85). After 1 month the real-time PCR assay detected 10^6 *bph*C gene copies g^{-1} soil for each of the biosensor strains. Gfp fluorescent cells were difficult to detect in these samples indicating a low level of PCB biodegradation. By the second month, the biosensor cell numbers had fallen below detectable limits using the plate count method. While real-time PCR could detect the biosensors at months 2 and 3, an exact determination of their numbers could not be estimated as the values were below the lower end of the linear range of the standard curve (10^3 *bph*C gene copies g^{-1}) (data not shown). No *gfp* fluorescent cells could be detected in these samples.

In the microcosms containing biosensor inoculated pea plants, both of the biosensor strains were detected in the rhizosphere soil and on the plant roots at each of the three monthly sampling times (**Fig. 3A**). The plate counts of F113L::1180*gfp* and F113rifPCB*gfp* on the roots were significantly higher than those in the rhizosphere soil during the three sampling times (**Fig. 3B**). By the third month, counts of these two strains in the rhizosphere soil were found to be significantly lower than those in the first month (by ~1 log). These results were consistent with the *bph*C copy number detected by the real-time PCR assay. Both F113L::1180*gfp* and F113rifPCB*gfp* strains were detected at levels of 10^6–10^7 *bph*C copies g^{-1} root at each of the sampling times (**Fig. 4**). There were no significant difference in TVC levels considering all variables (sampling time or biosensor strain) as determined by a one way ANOVA analysis.

Epi-fluorescent microscopy of pea roots inoculated with either F113rifPCB*gfp* or F113L::1180*gfp* showed high numbers of *gfp* expressing cells on the root surface and in the rhizosphere soil (**Fig. 5**). The cells were observed to form micro-colonies and to reside within the intercellular groves of the root epidermal cells. Since expression of the *gfp* in these two strains is driven by a chlorobenzoate inducible promoter, the fact that they were expressing the *gfp* would suggest there was active degradation of PCBs occurring within the microcosms. This degradation was likely to be due to the degradation activity of the F113rifPCB*gfp* or F113L::1180*gfp* strains themselves. However, as the soil and pea seeds were not sterilized prior to adding these inocula, PCB degradation and the formation of chlorobenzoate derivatives by indigenous microbes cannot be ruled out. No *gfp* expressing cells were visualized on plant roots or in the rhizosphere of plants growing in uncontaminated soil.

Table 1. PCB concentration in the soil/sludge samples

PCB congener	Soil A ppb*	Soil B ppb*	Soil C ppb*	Soil D ppb*
Mono-Chlorinated				
PCB1	0.61	0.96	5.6	<0.14
PCB2	0.52	1.6	0.56	<0.14
PCB3	40	2.0	3.3	<0.14
EPA marked congeners				
PCB 28 + 31	2200	165000	234	0.46
PCB52	4800	37000	60	0.21
PCB101	65000	8700	27	0.55
PCB118	32000	19000	58	0.28
PCB138	153000	2800	15	1.1
PCB153	299000	3500	18	1.7
PCB180	350000	1700	7.7	2.5
Groups of PCBs				
Mono-PCBs	42	4.5	9.4	<0.42
Di-PCBs	1500	4000	141	1.4
Tri-PCBs	5200	300000	440	0.92
Tetra-PCBs	20000	485000	557	1.5
Penta-PCBs	255000	90000	263	2.0
Hexa-PCBs	1300000	16000	79	6.8
Hepta-PCBs	1480000	7200	28	8.2
Octa-PCBs	400000	738	7.1	2.0
Nona-PCBs	26000	48	7.0	0.33
Deca-PCBs	320	1.4	2.4	None
Total PCBs	3490000	903000	1534	23

*ppb, parts per billion.

Table 2. Percentage of total number of F113L::1180*gfp* biosensor cells and *gfp*-expressing cells in the beads in microcosm samples

	A	B	C	D
Number of biosensor cells (expressed as a % of TVC)*	80	75	75	78
Numbers of *gfp*-expressing biosensor cells (expressed as a % of TVC)**	35	20	8	0.007
Percentage of *gfp*-expression cells of the total biosensor cells	43.8	26.7	10.7	0.01

*Determined from plate counts. **Determined from microscopy counts.

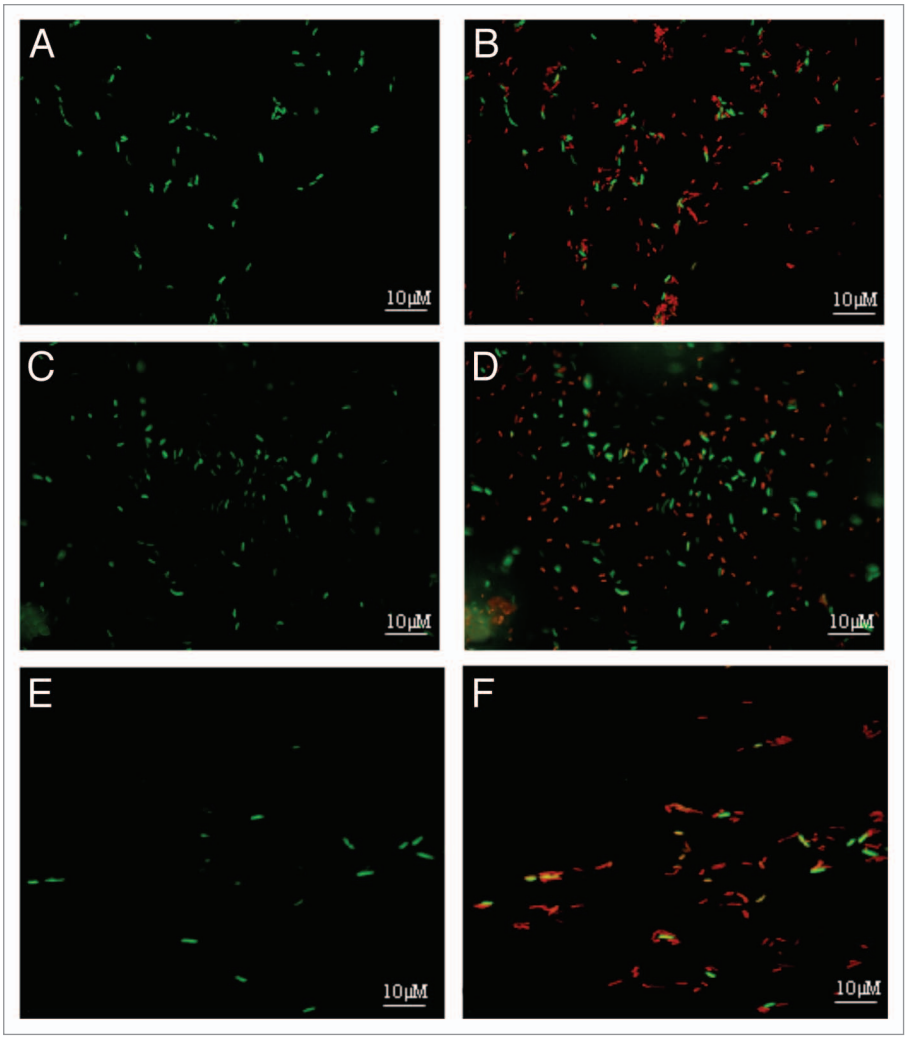

Figure 2. Alginate encapsulated biosensor (F113L:1180*gfp*) in PCB contaminated microcosms. Green cells are the *gfp* expressing biosensor cells, Red cells are biosensor cells not expressing *gfp* and non-biosensor cells. Images (A and B) were taken from the soil sample (B); Images C and D were taken from the sample (A); Images (E and F) were taken from sludge sample (C). Images (A, C and E) were taken using Gfp filter with excitation wavelength 465–495 nm; Images (B, D and F) were taken under filter with excitation wavelength 450–490 nm. Cells were counter-stained with 0.1% acridine orange. (X1000).

PCB remediation capacity is increased in the presence of biosensor strains. At the end of the experiment total PCB residues from each of the treatments were determined (**Fig. 6**). Since samples were analyzed only once it was not possible to test the significance of the results obtained. However, the direct inoculation of the PCB degrading biosensors (F113L::1180*gfp* and F113rifPCB*gfp*) into the PCB contaminated soil has led to reductions in total PCB content when compared with un-inoculated contaminated soil. The presence of un-inoculated pea plants did not result in the removal of any PCBs. Pea plants inoculated with the PCB degrading biosensors (F113L::1180*gfp* and F113rifPCB*gfp*) strains removed more PCBs than when these strains were directly inoculated into the soil. The highest total PCB removal was found in pots with pea plants inoculated with F113L::1180*gfp* (38.7%), followed by F113rifPCB*gfp* (32.8%).

Discussion

Boldt et al. reported using a F113rifpcb*gfp* biosensor to detect the biodegradation of 3-CBP and root colonization in 3-CBP spiked soil.[16] However, when this biosensor was directly inoculated into 3-CBP spiked soil only 1% of the biosensor cells were shown to be GFP fluorescent. They suggested that the presence of CBA degrading bacteria in the soil could affect the accuracy of the biosensor. Previous studies showed that by immobilising the biosensor cells, ease of detection, accuracy and reproducibility could be improved.[17-20] Thus, an alginate bead immobilized system for the delivery and examination of the biosensors was developed.

When the immobilized PCB degrading biosensors (F113L::1180*gfp* and F113rifPCB*gfp*) were introduced into PCB contaminated soil and sludge samples there was a positive correlation between the PCB levels within the samples and the percentage of biosensor cells that were fluorescent. The higher the PCB level in the sample, the more fluorescent cells that were observed. Greater number of fluorescent cells, indicated greater bioavailability of PCBs and their degradation by the GM biosensors which in turn indicated that the soil/sludge posed a greater risk to human health. However, in order to confirm the statistical significance of this correlation further study is needed, where the PCBs levels are determined more accurately and more samples are analyzed.

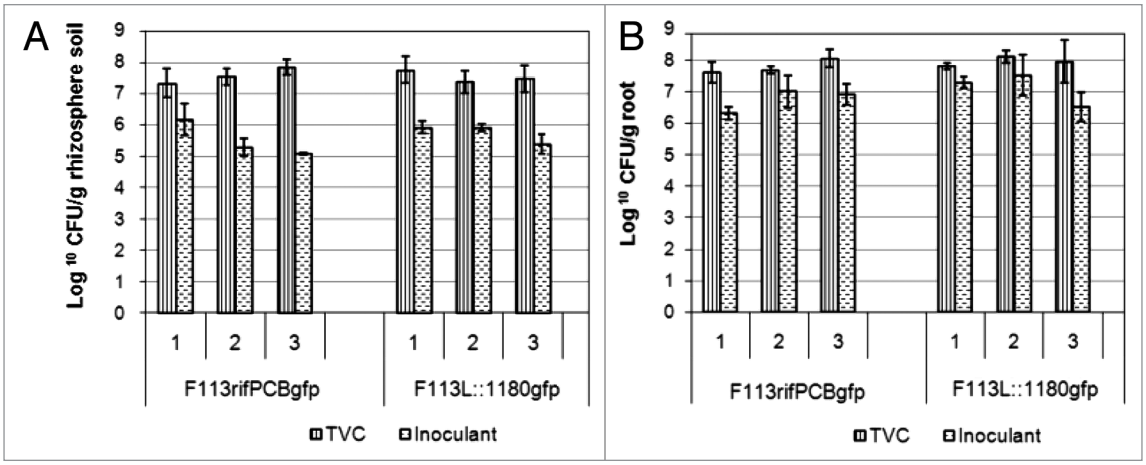

Figure 3. TVC and biosensor cell counts in (A) the rhizosphere soil and (B) on the roots of inoculated pea plants (numbers 1, 2 or 3 denote sampling month).

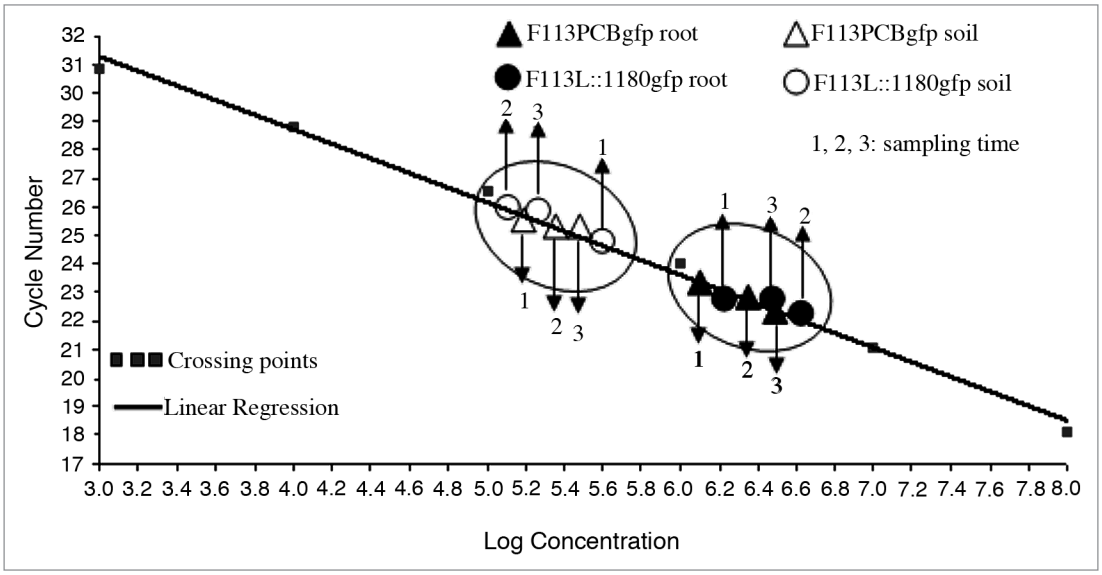

Figure 4. Real-time PCR detection of *bphC* gene copies from the root and rhizosphere soil samples of pea plants inoculated with F113rifPCB*gfp* and F113L::1180*gfp*. (Numbers 1, 2, 3 denote sampling month.)

When the non PCB degrading biosensor F113rif*gfp* was introduced into the same contaminated soil and sludge samples there were very few fluorescent cells observed. This result would suggest that there was little CBA present within the soil/sludge samples to induce *gfp* expression. There may be two possible explanations for this; there was little or no PCB degradation within the soil/sludge samples by the indigenous microbial population. Or the indigenous microbial communities rapidly degrade the CBA compounds released from PCB degradation. Chemical analysis of the samples and the microcosm experiments in this study would suggest that there is no natural PCB degradation occurring in the samples.

The results from this microcosm study showed that both F113L::1180*gfp* and F113rifPCB*gfp* survived for less than two months in unplanted soil. However, these strains maintained high population sizes in contaminated soils planted with pea even after 3 months. These large populations in turn resulted in degradation of PCBs within each of the treatments. Demnerova et al. carried out PCB degradation experiments using three plants species.[21] Their results showed that the number of natural PCB degraders on roots was at least ten times higher than those in bulk soil. Gfp expressing biosensor cells were easily detectable and much more numerous in soils planted with pea, indicating increased PCB degradation activity compared with unplanted soil.

The real-time PCR results correlated well with the total viable plate count results. Biosensor cell numbers detected by using qRT-PCR were 10-fold higher than the numbers detected using the plate counting method. This is due to the sensitivity of real-time PCR; it could detect not only the culturable inoculants, but also detects the non-culturable and dead inoculants.[22] It was also documented by Wang et al. that using a qRT-PCR method, a

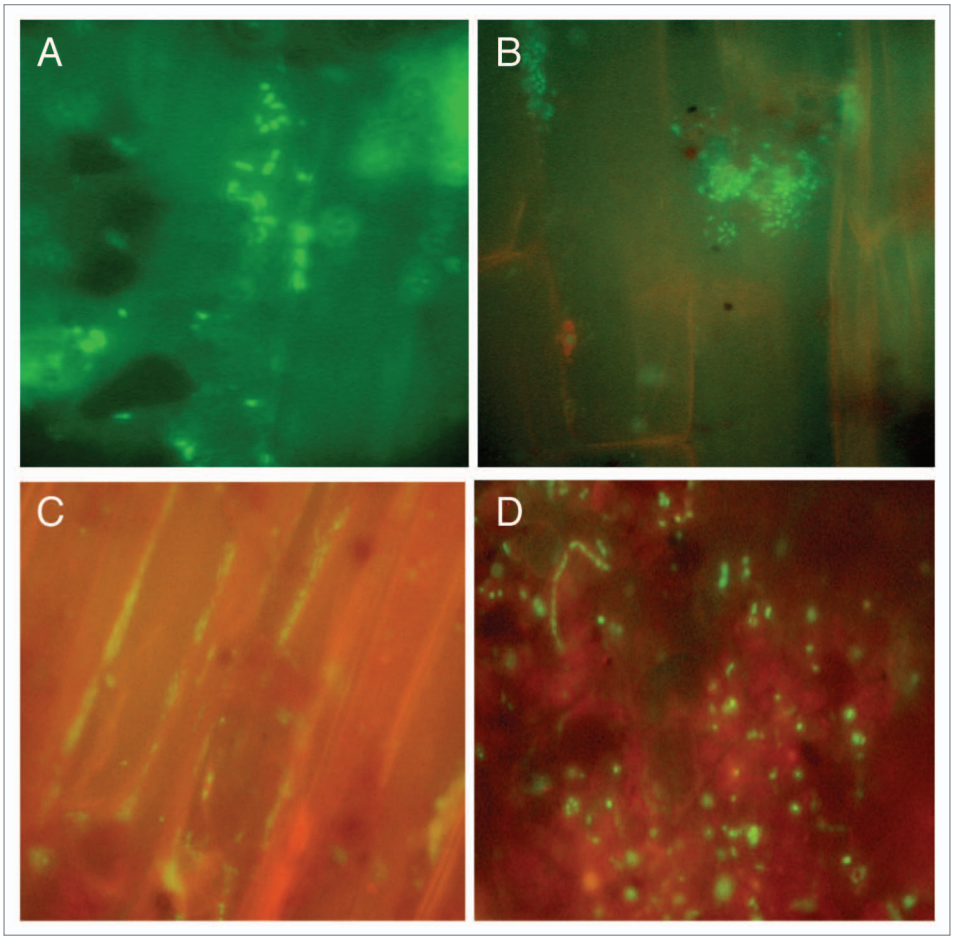

Figure 5. Visualization of F113 PCB biosensors reporting PCB degradation in the rhizosphere of inoculated Pea plants. (A and B) F113L::1180*gfp* colonizing the root surface of inoculated Pea plants. (C) F113rifPCB*gfp* colonizing the root surface of inoculated pea plants. (D) F113L::1180*gfp* in rhizosphere soil expressing *gfp*. (x1000).

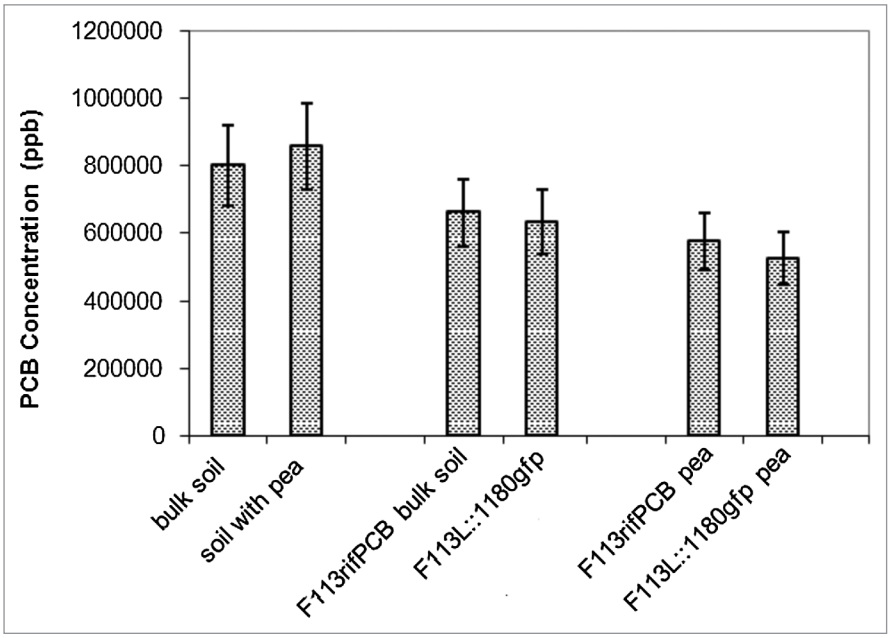

Figure 6. Total PCB residues in each treatment 3 months after inoculation with PCB degrading strains (bars represent the mean error of the method).

Table 3. Bacterial strains used in this study

Bacteria	Characteristics	Reference
P. fluorescens F113rif	Spontaneous Rif resistant mutant of F113.	11
P. fluorescens F113rif*gfp*	Wild type *P. fluorescens* F113; Km^r-Pm::*gfp*mut3	14, 29
P. fluorescens F113rifPCB*gfp*	*P. fluorescens* F113rifpcblac3.1. *bph* promoter-lacZ fusion; Bp$^+$; Rif^r, Km^r-Pm::*gfp*mut3	14, 29
P. fluorescens F113L::1180*gfp*	Bp$^+$; Nodbox *bph* fusion; lac$^+$; Rif^r, Km^r-Pm::*gfp*mut3	14, 29

Rif^r, rifampicin resistance; Km^r, Kanamycin resistance; *gfp*, green fluorescent protein.

10-fold higher level of the engineered *Pseudomonas putida* could be detected compared to the plate counting method, during 2-chlorobenzoate degradation in soil.[23]

PCB removal was observed in all inoculated samples. The significance of these results could not be tested as each sample was analyzed just once. However, the trend did seem to indicate that PCBs were remediated more efficiently within the pea planted microcosms than in inoculated unplanted soil samples. Within the planted samples, between 30–42% total PCBs were removed, with F113L::1180*gfp* being the most efficient strain. F113L::1180*gfp* was constructed by the insertion of rhizobial nodulation promoters (nodbox) and its regulatory systems upstream of the *bph* operon to drive operon expression.[12,24] The PCB degradation rates of F113L::1180*gfp* observed in the current study is presumed to be due to this higher expression *bph* operon expression in the pea rhizosphere.

Both biosensors F113rifPCB*gfp* and F113L::1180*gfp* reported the degradation of PCBs in the soil and rhizosphere of inoculated microcosms, through expression of the Gfp protein. Since expression of *gfp* in these strains is dependent on the presence of PCB degradation end products it indicates that there was active degradation of the PCBs. Liu et al. had previously shown the usefulness of these biosensor strains to detect PCB degradation using in vitro assays.[14] This current study demonstrates that these biosensors can be used to evaluate a specific contaminated site for bioremediation potential as well as to monitor PCB degradation in real PCB contaminated soil.

Materials and Methods

Bacterial strains. The bacterial strains used in this study are detailed in **Table 3**. These strains were maintained on LB agar supplemented with either kanamycin or rifampicin.[25] PCB degrading strains were also maintained on minimal media supplemented with biphenyl as the sole carbon source.[26]

Construction of CBA/PCB biosensor strains. The construction and characterisation of the biosensor strains are described in Liu et al.[14] Briefly, the pJBA26 plasmid carrying the mini-Tn5-Km^r-Pm::gfpmut3-T0-T1 transposon was introduced into *P. fluorescens* F113 derivatives (F113rif, F113RifPCB and F113L::1180) by triparental matings.[15] The selection plates were LB supplemented with Rif and Kan. After mating, the transconjugant colonies were purified and tested by spraying with 2,3-dihydroxybiphenyl to detect the presence of an active *bphC* gene product, thereby confirming the presence of the *bph* operon. The presence of the biosensor transposon was also determined by PCR detection of the *gfp* gene. The number of insertion events was determined by Southern blot analysis and the effect of the insertion on the host strain was evaluated by comparison of growth rates and Biolog® phenotypic profiles with the parental strain.

Encapsulation of biosensor strains in alginate beads. The biosensor bacterial cells were grown overnight in minimal media broth supplemented with 10 mM sodium citrate. The cultures were centrifuged at 8,000 rpm (7,650 g) at 4°C for 15 min and then washed twice in 0.9% sterile saline solution, centrifuged and resuspended in sterile saline Ringer's solution and adjusted to an absorbance of 0.8. The cells were mixed with sterile 3% sodium alginate at a ratio 1:4. The mixture was then extruded through a 5 ml sterile syringe into a sterile 2% calcium chloride solution to form beads of approximately 1 mm in diameter. For cell enumeration and fluorescent cell detection, 30 beads were dissolved in 10 ml of Na_2PO_4 buffer (pH 7.0), followed by vortexing for 5 min. Total variable cells (TVC) counts were carried out by serial dilution and plated on nutrient agar plates while biosensor strains were counted on nutrient agar amended with Rif + Kan. There were approximately 5 x 10^6 biosensor cells bead^{-1} after the encapsulation.

Evaluation of alginate bead encapsulated biosensors in PCB contaminated environmental samples. Three PCB contaminated matrixes were used to evaluate the biosensors. The PCB content of these samples varied from low to medium to high. The high PCB content sample (A) was a contaminated sand from Australia. The medium PCB content sample (B) was a contaminated soil from Lhenice Czech Republic (reviewed in refs. 8 and 27) and low PCB content sample (C) was a contaminated sludge sample from the Hudson River in the USA. As a control sample, field soil from the grounds at I.T. Carlow which had a very low or background level (0.023 ppm) of PCBs was used. Biosensor (either F113L::1180*gfp* or F113rif*gfp*) encapsulated beads (30 per 100 grams of soil) were introduced into samples from each of the soil/sludges. Non-encapsulated biosensor strains were also inoculated (1 x 10^8 free cells g^{-1} soil) into another set of samples from each of the soil/sludges. Triplicate samples were set up for each treatment. The percentage of *gfp* expressing cells in the alginate beads was examined by epi-fluorescent microscope. TVC and biosensor cells were counted by plate counting.

The use of PCB Biosensor strains in rhizoremediation studies. Samples from the PCB contaminated sample A were used to prepare small scale microcosm studies. For this microcosm study the A sample was mixed (1:3) with the Carlow soil (D) (which had been autoclaved twice at 121°C at 15 p.s.i for 2 h).

The microcosms were set up by adding 120 g of this soil mixture to aluminium pots. Cultures were grown at 30°C in LB broth overnight. The cultures were centrifuged at 8,000 rpm at 4°C for 15 min and re-suspended in sterile Ringer's solution and adjusted to an $OD_{600\ nm}$ 4.0. Two sets of experiments were carried out. The first set involved the direct inoculation of the biosensors into the PCB contaminated soil mixture. Here the PCB soil was inoculated at a rate of 0.1 ml inoculum g^{-1} soil. The second set of experiments was carried out using inoculated pea plants [*Pisum sativum* var Early Onward (Suttons, UK)]. Pea seeds were soaked in sterile water overnight and then coated with one of the inoculants (20 µl inocula per seed). The seeds were allowed to dry for 30 min before they were planted into pots containing 120 g of the PCB contaminated soil mixture (five seeds/pot). Nine pots were set up for each bacterial strain. Control pots containing un-inoculated soil or un-inoculated seeds were also included. All pots were held at 20–25°C under a 16 h light/8 h dark regime and watered once a week with 60 ml of sterile water. Triplicate samples from each treatment were analyzed for total viable counts (TVC) and inoculum number. Only at the end of the 3 months were samples taken for PCB determination.

Enumeration of inoculants within soil and plant rhizosphere. Bacterial stains were enumerated within the soil and plant rhizosphere using both the standard plate count method and a real-time PCR assay.

Samples were taken from triplicate pots once a month over three months. For the planted pots, samples were taken from two locations within each pot: (1) soil from the rhizosphere and (2) from the plant root itself. A sample of the root was aseptically removed from the plants and homogenized using a sterile pestle and mortar. For the unplanted pots, 1 g of bulk soil was taken from each pot. 1 g of the homogenized root tissue, rhizosphere soil or bulk soil sample was vortexed for 10 min and then serially diluted in sterile Ringers solution. To determine the TVC, 100 µl of these dilutions were spread plated onto NA plates, which were incubated at 30°C for 72 h and the resulting colonies were counted. To enumerate the inoculum levels these colonies were sprayed with 2,3-dihydroxybiphenyl (2,3-OHBP). Colonies which developed a bright yellow colouration (indicating the presence of the *bph* operon) were counted and presumed to represent the inoculum level within the sample.[14]

The real-time PCR assay was carried out using SYBR Green chemistry and the Roche Lightcycler® (Roche). DNA was extracted from triplicate samples using the UltraClean Soil DNA extraction kit (MOBIO Laboratories Inc., Cambio Ltd., UK) according to the manufacturer's instructions. The target gene was the *bph*C gene. Since there is only one copy of this gene within the *bph* operon, and one operon per inoculum cell, then one gene copy was assumed to represent one bacterial cell. The primers used were as follows: LCC forward 5'GCG TTT TAT ACC GAC GTG CT3' and LCC reverse 5'AGC ATG AAG TGA TGA ATG CG3'.[27] Each reaction consisted of 2 µl DNA sample, 3 µl $MgCl_2$ (25 mM), 1 µl of each primer (10 µM), 10 µl of SYBR Green master mix (Sigma), 2 µl BSA (10 mg ml^{-1}) and 2 µl sterile de-ionised water. The annealing temperature used was 62°C for 5 seconds. Quantification of PCR product was carried out by comparing the fluorescence of the PCR product of samples with the fluorescence of several dilutions of a specific external standard (F113rifpcb DNA).

Determination of PCB concentrations within soil. At the end of the experiment, soils in pots inoculated under the same conditions were pooled for the purposes of chemical analysis. The soils were mixed thoroughly, placed into glass containers and then sent to be analyzed using gas chromatography by an independent commercial testing laboratory (Ecochem a.s., Czech Republic) using USEPA method 1668, details of which can be found on their website (accessed April 2010 http://engecochem.ample.cz/index.jsp). sample was analyzed once.

Epi-fluorescent microscopy. Biosensor cells were visualized under a Nikon E400 epi-fluorescent microscope equipped with a 100 W mercury short arc photo-optic lamp and two filters with the excitation wavelength of 465–495 nm and 450–490 nm, respectively. Lucia® imaging software (version 4.6) was used to capture and process microscopic images. To visualize the biosensor cells within the alginate beads, the beads were washed and then sliced into thin sections. These sections were then placed onto a microscope slide, crushed and examined under the microscope for *gfp* expressing cells. Total bacterial counts were determined by staining the sample with 0.1% acridine orange for 1 min which stained the bacterial cells red. Biosensor visualization in plant tissue was achieved by staining the tissue section in either 0.05% methyl violet for 30 s or 0.1% acridine orange for 1 min, which caused the plant cells to fluoresce red under near UV light.

Statistical analysis. All experiments (except PCB analysis) were performed using three replicates. The results shown are the averages of triplicate sampling for error analysis. Standard deviations are shown as error bars within the graphs at the $p < 0.05$. Data was subjected to one way ANOVA or Students-t tests to determine significance at the $p < 0.05$. Principle Component Analysis (PCA) was performed using Minitab.

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