

Enhancement of Plant-Microbe Interactions Using a Rhizosphere Metabolomics-Driven Approach and Its Application in the Removal of Polychlorinated Biphenyls^{1[w]}

Kothandaraman Narasimhan, Chanbasha Basheer, Vladimir B. Bajic, and Sanjay Swarup*

Department of Biological Sciences (K.N., S.S.) and Department of Chemistry (C.B.), 10 Science Drive 4, National University of Singapore, Singapore 117 543; and Institute for Infocomm Research (I2R), 21 Heng Mui Keng Terrace, National University of Singapore, Singapore 119 613 (V.B.B.)

Persistent organic pollutants, such as polychlorinated biphenyls (PCBs), are a global problem. We demonstrate enhanced depletion of PCBs using root-associated microbes, which can use plant secondary metabolites, such as phenylpropanoids. Using a “rhizosphere metabolomics” approach, we show that phenylpropanoids constitute 84% of the secondary metabolites exuded from *Arabidopsis* roots. Phenylpropanoid-utilizing microbes are more competitive and are able to grow at least 100-fold better than their auxotrophic mutants on roots of plants that are able to synthesize or overproduce phenylpropanoids, such as flavonoids. Better colonization of the phenylpropanoid-utilizing strain in a gnotobiotic system on the roots of flavonoid-producing plants leads to almost 90% removal of PCBs in a 28-d period. Our work complements previous approaches to engineer soil microbial populations based on opines produced by transgenic plants and used by microbes carrying opine metabolism genes. The current approach based on plant natural products can be applied to contaminated soils with pre-existing vegetation. This strategy is also likely to be applicable to improving the competitive abilities of biocontrol and biofertilization strains.

The establishment of large numbers of metabolically active populations of beneficial soil microbes is critical for the success of several environmental remediation and agricultural practices (Metting, 1992). These microorganisms are successful in getting established in the soil ecosystem due to their high adaptability in a wide variety of environments, their faster growth rate, and their biochemical versatility to metabolize a variety of natural and xenobiotic chemicals. Majority of the microbial population found in the soil is associated with the plant roots, where their numbers can reach up to 10^9 to 10^{12} per gram of soil (Whipps, 1990), leading to a biomass equivalent to 500 kg ha^{-1} (Metting, 1992). This abundance in vegetated soils is due to the availability of nutrients via plant root exudation (Brimecombe, 2001), which can stimulate microbial growth in the immediate vicinity of the roots (a region also known as the “rhizosphere”). Hence, the rhizosphere has been promoted as the ideal site to modify microbial

populations (“rhizoengineering”) to suite various applications in the soil (O’Connell, 1996).

Rhizoengineering has been demonstrated successfully by devising strategies that favored the growth of the targeted microbes that possessed the ability to metabolize exotic nutrients exuded by plants (Lugtenberg, 2001). It was thus possible to create a nutritional bias that can be especially successful in identifying microbial populations due to the general nutrient-limiting conditions in the rhizosphere. One of the earliest successes in rhizoengineering was based on favorably partitioning the exotic nutrient, opines, which were produced by the transgenic plants (Oger, 1997; Savka and Farrand, 1997). This led to the improved and competitive growth of the metabolizing strains in comparison with the microbes unable to metabolize opines. Success of the above approach is contingent upon the general nutrient-limiting conditions that prevail in the rhizosphere. Studies have shown that such conditions are prevalent in the rhizosphere of many plant species, which leads to starvation of root-associated bacterial (“rhizobacterial”) populations (Normander, 1999).

We reasoned that it might be possible to rhizoengineer by partitioning the nutrients with complex structures produced naturally by the wild-type plants without having to rely on transgenic plants, whose establishment in contaminated soils may pose practical limitations. We have therefore investigated the role of the natural products with highly complex

¹ This work was supported by the Academic Research Fund, National University of Singapore (grant nos. R-154-000-064-112 and R-154-000-059-112). K.N. was supported by a Research Scholarship from the National University of Singapore.

[w] The online version of this article contains Web-only data. The supplemental material is available at www.plantphysiol.org.

* Corresponding author; e-mail dbsss@nus.edu.sg; fax 65-6779-2486.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.102.016295.

structures, the secondary metabolites, and we have carried out profiling of the root exudates to identify targeted compounds for creating the nutritional bias. For convenience, we have referred to this as "rhizosphere metabolomics." We have studied the utility of these exotic natural products for rhizoengineering purposes by establishing an *Arabidopsis-Pseudomonas* spp. rhizosphere model based upon the following considerations: (a) the identification of 125 secondary metabolites, including phenylpropanoids, in the root exudates of *Arabidopsis*; (b) the availability of near-isogenic lines of *Arabidopsis* mutants in secondary metabolism; (c) a *Pseudomonas* spp. (plant growth promoting rhizobacteria) strain (designated Pp-wt) that can efficiently colonize *Arabidopsis* roots, use phenylpropanoids, and independently degrade a major class of persistent organic pollutants, namely, the polychlorinated biphenyls (PCBs); and (d) the availability of a phenylpropanoid auxotrophic mutant (designated Pp-mut) of the above strain whose colonization and PCB-degradation traits are unaffected with respect to the Pp-wt. The three properties of the Pp-wt strain were critical in establishing this experimental model.

Using the above model, we show that plant secondary metabolites are exuded in sufficient amounts to establish a rhizosphere biased to a rhizobacterial strain that is capable of metabolizing phenylpropanoids. In this regard, we also apply the rhizoengineering method for significantly enhanced removal of PCBs, which rank sixth in the list of hazardous substances (Agency for Toxic Substances and Disease Registry, 2001), and globally, PCBs are second only to benzene among the organic pollutants. The world production of PCB until 1988 was estimated to be 1.2 million tons. Of this, 31% is thought to be already in the environment, 4% has been destroyed, and 65% is still in use or in storage. Thus, more than twice the amount now in the environment is still available for future contamination (National Environment Indicator Series, 1996). PCBs are additionally hazardous due to their rapid movement in the ecosystem, their high persistence, their ability to accumulate in the food chain, and their toxicity to various organisms. Hence, approaches to enhance removal of PCBs and other contaminants are going to be critical for alleviating this problem in the near future. Using the proposed approach here, already vegetated soils can be targeted for rhizoengineering. This approach, therefore, complements the previous ones requiring establishment of transgenic plants (Oger, 1997; Savka and Farrand, 1997).

RESULTS AND DISCUSSION

Rhizosphere Metabolomics

Identification and quantitation of the array of phenylpropanoid compounds present in the root exudates is a prerequisite for choosing target compounds

that can be used for creating a nutritional bias in the rhizosphere. We have, therefore, analyzed the proportions of phenolic compounds in the root exudates of *Arabidopsis* to focus on the secondary metabolites. Phenylpropanoid compounds including lignins, coumarins, flavonoids, auronones, sinapates, and anthocyanins were identified as the most abundant class, which includes what we have used for creating a nutritional bias. Quantitative analyses of root exudates showed the total organic carbon content to be in the range of 14 ng mL^{-1} root exudate. Total phenolics constitute $7.0 \pm 0.1 \text{ ng mg}^{-1}$ root fresh weight, which in turn constitutes 50% of the total organic carbon content. Rhizosphere metabolomics using a combination of reverse phase (RP)-HPLC and electron spray ionization mass spectrometry (ESI/MS) analyses showed flavonoids as the major class (37%), followed by lignins (22%) in the root exudates (Fig. 1a). We have identified 149 hydrophobic compounds consisting of 125 secondary metabolites in the root exudates; this is the most comprehensive analysis of the rhizosphere metabolites for a single plant species to date. Of the 125 secondary metabolites, 76% belonged to the phenylpropanoid class of compounds (Fig. 1a). To have an experimental set up of *Arabidopsis* lines capable of accumulating phenylpropanoids to different levels, we have chosen mutants affected in the flavonoid biosynthetic pathway because these compounds formed the largest proportions (64%) of the phenylpropanoids. The mutants chosen were *tt4* (Pelletier, 1999), *ttg* (Walker, 1999), and *tt8* (Nesi, 2000) in the isogenic Landsberg *erecta* (*Ler*) ecotype background. Metabolic profiles of root exudates from the wild type and the three mutants showed a widespread effect of the mutations on secondary metabolism. The mutant *tt4* lacks the structural gene for chalcone synthase and does not accumulate flavonoids (Pelletier, 1999; Fig. 1, b and d). However, the exudates from *tt4* roots had an abundance of several other phenylpropanoid compounds. Interestingly, the mutant *ttg* (*ttg* gene encodes a WD40-repeat protein; Walker, 1999) accumulates both flavonoids and their conjugates to higher amounts in the roots. The mutant *tt8* (*tt8* gene encodes a basic helix-loop-helix type regulator; Nesi, 2000) largely accumulates aglycones of flavonoids (Pelletier, 1999; this study). Although, the three mutants are known to be affected in flavonoid metabolism, rhizosphere metabolomics revealed that several other phenylpropanoids are additionally affected (see supplementary information, which can be viewed at www.plantphysiol.org). For example, the following seven compounds were higher in *tt8* with respect to the levels in *ttg*; numbers in parenthesis represents X-fold higher levels: dihydromyricetin (9.25), 3-O acetyl-4'5', 7 tri O-methyl kaempferol (8.36), quercetin 3-rhamnosylglucoside (7.41), coumaric acid (7.78), sinapoyl tetra acetyl Glc (16.44), leucocyanidin (5.10), and leucodelphinidin (8.67).

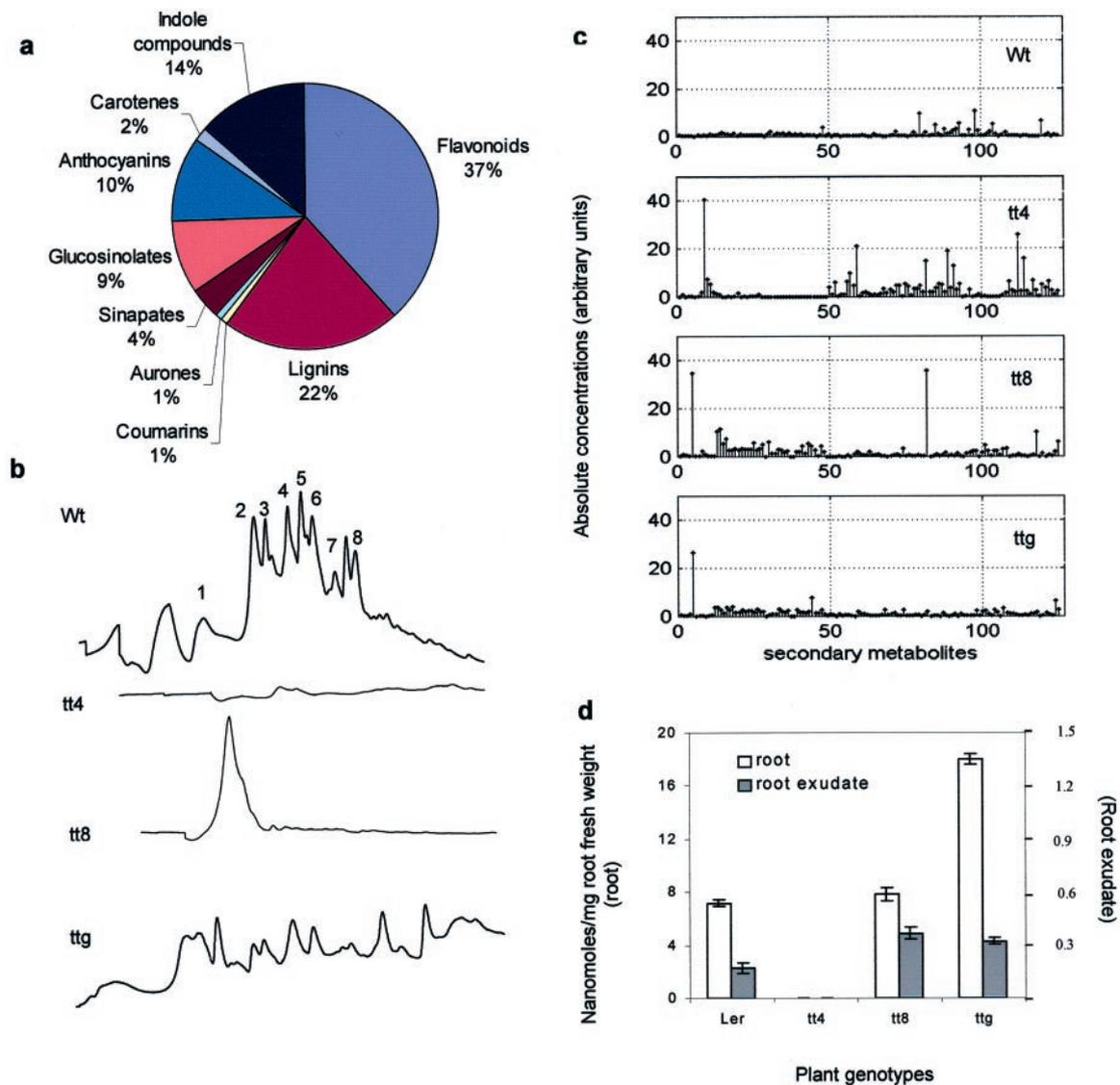


Figure 1. Rhizosphere metabolomics of Arabidopsis. a, Proportions of secondary metabolite classes in the root exudates of Arabidopsis, ecotype Ler. Secondary metabolites within each class identified by ESI/MS are listed in the supplementary information. b, RP-HPLC profiles of phenolic compounds present in the root exudates of four Arabidopsis plant lines: a, wild type; b, *tt4*; c, *tt8*; and d, *ttg*. Plant root exudates were harvested by washing roots of 20-d-old Arabidopsis plants grown on water agar plates that were kept in an inverted position as previously described in “Materials and Methods. RP-HPLC analysis was performed using previously described method (Pillai and Swarup, 2002). Fractions were analyzed by ESI/MS with and without acid hydrolysis to identify the aglycones and their conjugations. The eight peaks correspond to the following compounds: peak 1, quercetin glucoside; peak 2, quercetin rhamnoside; peak 3, kaempferol rhamnoside; peak 4, cyanidin glucoside; peak 5, chlorogenic acid; peak 6, kaempfero,3-O-galactoside; peak 7, indole compound; and peak 8, quercetin rhamnosyl glucoside. c, Rhizosphere metabolomics study of 125 secondary metabolites exuded by four Arabidopsis line roots. Intensity values obtained from the ESI/MS analysis for the individual compounds are presented in c, in the same scale for wild type and the three mutants, where position on the horizontal axis represents different secondary metabolites, as listed in the supplementary information. d, Quantitation of flavonoids in the roots and root exudates of Arabidopsis wild-type and metabolic mutant plants (*tt4*, *tt8*, and *ttg*). Quantitative analysis of the quercetin aglycone was carried out using selected ion monitoring (SIM) mode of the deprotonated molecule peak for acid hydrolyzed root exudates. Quercetin was identified as the major flavonoid aglycone in the root exudates of the three flavonoid producing lines.

Similarly, there were another seven phenylpropanoid compounds that were present at higher levels in *ttg* than in *tt8* plants: naringenin rhamnoside (22.30), pyro-Glu (6.16), 3,4-dihydroxybenzoic acid (8.22),

ferulic acid (6.46), syringic acid (12.35), cyanidin (6.16), and methyl IAA-Glc (10.29).

Comprehensive profiling of secondary metabolites in the rhizosphere showed an abundance of phenyl-

propanoids in the exuded compounds and significant qualitative differences of several compounds in the four lines studied (Fig. 1c; supplementary information). Interestingly, in *tt8* and *ttg*, the overaccumulation of quercetin, which is the major flavonoid of Arabidopsis, is not significantly different in the root exudates as it is within the roots (Fig. 1d). Quercetin is found in nanomole amounts in the root tissue, whereas it is present in almost picomole amounts in the root exudates. It is noteworthy that although a minor fraction of the flavonoids is exuded (Fig. 1d), it forms a high proportion (37%) of the secondary metabolites in the root exudate (Fig. 1a). On the basis of the above results, phenylpropanoids meet several criteria outlined earlier for serving as target nutrients for rhizoengineering: They are exuded by all plants, they are abundant in the rhizosphere, and they have complicated structures to qualify as being "unusual" nutrients for the rhizobacteria so as not to be easily metabolized by most rhizobacteria.

Rhizoengineering Based on Phenylpropanoid Compounds

To design a suitable plant-microbe pair for testing the efficiency of rhizoengineering, we set up an experimental model using the Arabidopsis wild-type and flavonoid metabolic mutant lines exuding different amounts of phenylpropanoid compounds, together with an efficient rhizocolonizing strain of *Pseudomonas* spp. (utilizer and nonutilizer strains for phenylpropanoids). The *Pseudomonas putida* PML2 (designated as Pp-wt here; Pillai and Swarup, 2002) was specially chosen for its ability to utilize various phenylpropanoid compounds, which form the majority of the exuded secondary metabolites (Fig. 1a). To study the effect of phenylpropanoid utilization on colonization and competition abilities of rhizobacterial strain, an auxotrophic mutant (Pp-mut) derived from Pp-wt was used. Over a 28-d period, Pp-wt consistently better colonized the roots of the flavonoid-producing Arabidopsis genotypes (*Ler*, *tt8*, and *ttg*) than the roots of flavonoid null mutant Pp-mut (Fig. 2a). On the roots of the three flavonoid-producing plants, higher colonization levels were clearly visible with green fluorescent protein (gfp)-tagged Pp-wt cells (data not shown). Occurrence of cells adjacent to each other in groups of two or more indicated that active cell division was taking place on the root (Bloemberg, 1997), validating a successful colonization. As previously reported, colonizing bacteria were present mainly along the furrows of the root epidermal cells surface (Bloemberg, 1997). On the basis of plate counts, there were no significant differences in the colonizing ability of Pp-wt on roots of the three flavonoid-producing Arabidopsis lines. However, slightly higher colonization levels by the gfp-tagged Pp-wt were seen on the flavonoid-overproducing strains *ttg* and *tt8*. This was more

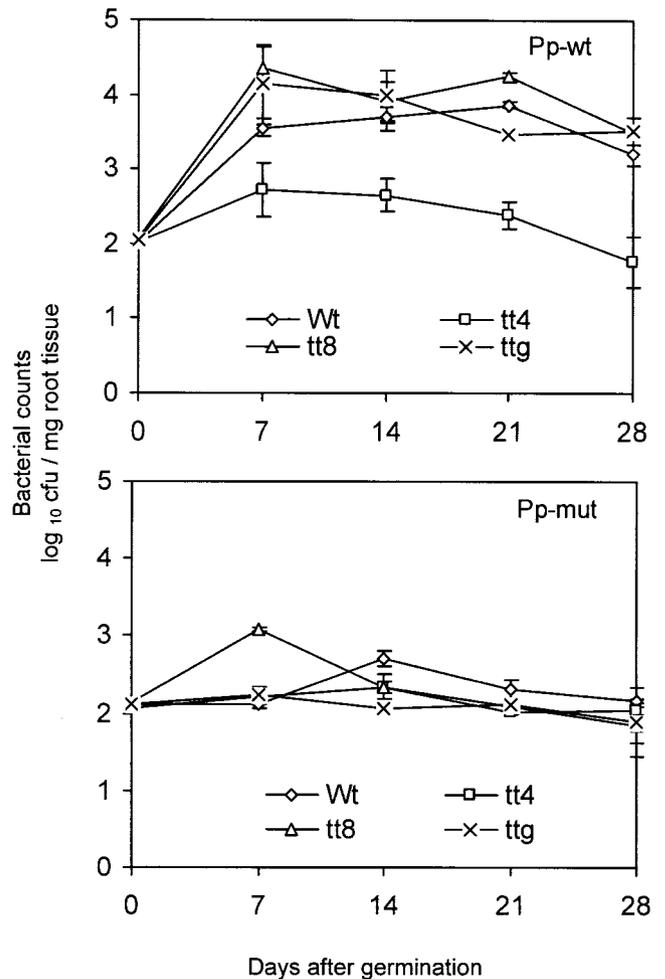


Figure 2. Growth of *P. putida* strains on Arabidopsis wild type and three flavonoid metabolic mutants *tt4* (null mutant), *tt8*, and *ttg*, (flavonoid overproducers). Seeds were inoculated individually with cultures of phenylpropanoid utilizer Pp-wt (a) or phenylpropanoid auxotrophic mutant Pp-mut strain (b). Bacterial counts were determined by dilution plating samples consisting of roots and tightly adhering soil. Vertical bars represent SE. Experiments were repeated at least three times.

evident on the upper portions of the roots, from where higher exudation has been reported (Lugtenberg, 2001). Although the population of Pp-wt increased by almost two orders of magnitude on these flavonoid-producing lines (*Ler*, *ttg*, and *tt8*), there was no significant colonization by Pp-wt on the roots of *tt4* plants, which do not produce flavonoids (Fig. 2a). In agreement with results based on plate counts, confocal microscopy also confirmed lower colonization levels of gfp-tagged Pp-wt on the roots of *tt4* plants (data not shown). In comparison, the phenylpropanoid-auxotrophic mutant strain (Pp-mut) was unable to colonize any of the four Arabidopsis lines used in this study (Fig. 2b). These differences in the colonization ability of the wild-type and auxotrophic mutant *Pseudomonas* spp. strains on the four Arabidopsis lines therefore showed that the

presence of higher levels of phenylpropanoids led to higher levels of bacterial populations. The phenylpropanoid-utilizer strain, therefore clearly showed a nutritional advantage on the flavonoid-exuding plants.

Next, we studied the abilities of the phenylpropanoid-utilizer strain and its auxotrophic mutant to compete for colonization on the four *Arabidopsis* lines. In the presence of Pp-mut, the Pp-wt strain was consistently able to better colonize the three flavonoid-producing *Arabidopsis* lines wild type, *tt8*, and *ttg*. On these *Arabidopsis* lines, Pp-wt showed higher growth levels than Pp-mut by \log_{10} to 1.5 units, clearly indicating a better competitive ability of Pp-wt over Pp-mut. This higher colonization ability of Pp-wt in a competitive situation diminished by 28 d after germination in the wild-type *Arabidopsis* plants (Fig. 3a), whereas such an advantage persisted on *tt8* and *ttg* plants (Fig. 3, c and d), which exuded double the amount of flavonoids (Fig. 1d). The decrease in colonization of wild-type *Arabidopsis* roots after 21 d could be due to exhausted carbon source once the bacterial population reached its maximal size, whereas the growth of bacteria in the rhizosphere of the plant mutants, which secrete double amount of root exudates (Fig. 1d), could be maintained for a longer time. Alternatively, the decline after 21 d of the bacterial growth on wild-type roots could be due to a rapid decrease in the phenylpropanoid exudation from the roots of wild-type plants in comparison with that from the phenylpropanoid-overproducing mutants.

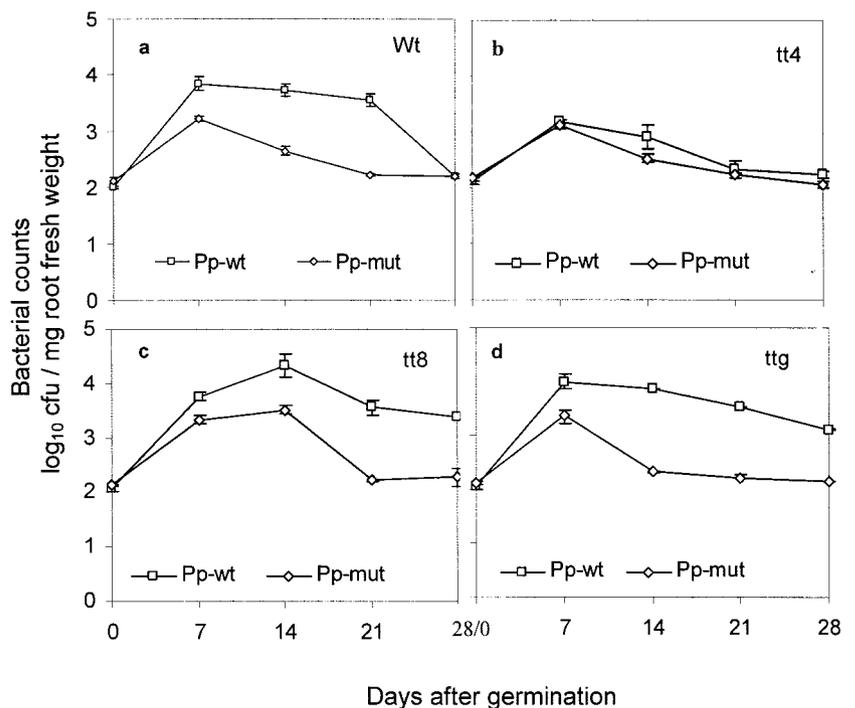
Comparison of the growth of Pp-mut strain in colonization (Fig. 2b) and competition (Fig. 3b) showed that it was better able to colonize roots of flavonoid-

producing plants after mixed inoculations. The presence of Pp-wt cells somehow improved the growth of Pp-mut strain slightly albeit for a short period. These observations suggested some form of cross feeding, perhaps of metabolic intermediates, as reported in several other cases of mixed inoculations (Dekkers, 2000).

Rhizoengineering-Based Improvement of PCB Removal

The last set of experiments address the question of whether rhizoengineering based on plant secondary metabolites can lead to an improvement in the removal of persistent organic pollutants, such as PCBs, from soils. Although PCB-degrading bacteria are found ubiquitously in the environment, a majority of them are inefficient in degrading PCBs (Donnelly, 1994). Major cause for this seems to be the lack of sustaining nutrients in the near-starvation conditions found in the soils, including the rhizosphere (Normander, 1999). Hence, the primary challenge for successful bioremediation of PCB-contaminated soil is to devise methods to encourage the growth (leading to more efficient PCB-removal) of a select species of microbes, which either are indigenous to PCB-contaminated sites or are introduced to these sites. Hence, for our present studies, the dual properties of the Pp-wt strain to use phenylpropanoids as nutritional sources (Pillai and Swarup, 2002) and its ability to degrade PCBs (Fig. 4) were a key element for testing our hypothesis. Liquid cultures of Pp-wt containing three PCBs, Aroclor, 4Cl-PCB, and 2Cl-PCB ($53 \mu\text{M}$ each), allowed maximal microbial growth to be achieved in 96 to 120 h. Both Pp-wt and Pp-mut

Figure 3. Competition experiments using *Arabidopsis-Pseudomonas* spp. model. Growth is shown for the phenylpropanoid utilizer (Pp-wt) and the auxotrophic mutant (Pp-mut) strains following mixed inoculations on seeds of *Arabidopsis* wild type and three flavonoid mutants (*tt4*, *tt8*, and *ttg*). Bacterial counts were determined as described in Figure 2 legend. Five randomly chosen plants were used as replicates for each sample. Vertical bars represent SEs. Experiments were repeated at least three times.



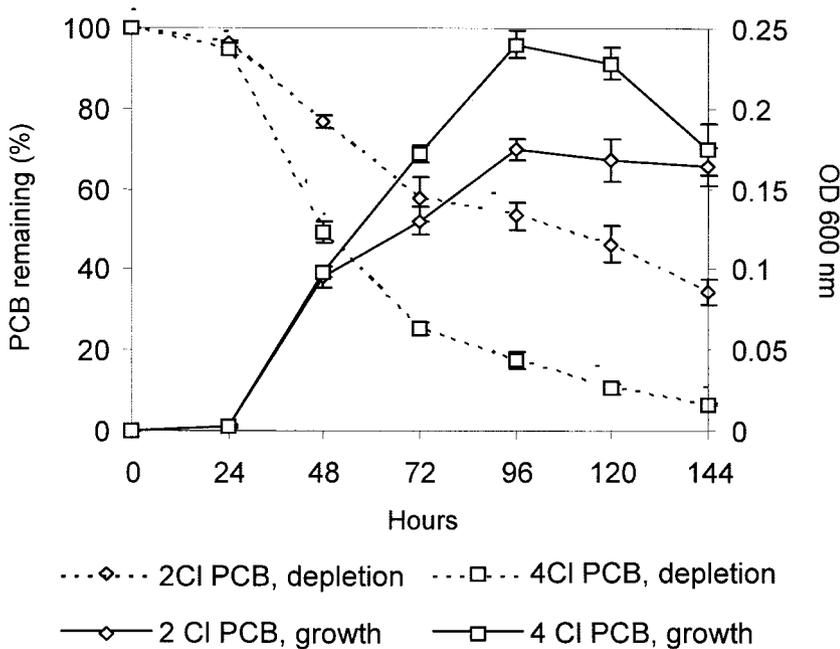


Figure 4. PCB depletion by *Pseudomonas* spp. strains in liquid cultures. Minimal medium supplemented with 0.5% (w/v) glycerol and 10 ppm of 2CI-PCB or 4CI-PCB was inoculated with culture of the phenylpropanoid-utilizing Pp-wt strain, and bacterial growth was measured at A_{600} . PCBs were quantified using gas chromatography (GC)/MS analysis in the SIM mode. Five replicates were used for each sample, and the experiments were repeated at least three times. Vertical bars represent SEs. Experiments were repeated at least three times.

had comparable growth in the different PCBs used in the study (data not shown). Depletion was quantified for 2CI-PCB and 4CI-PCBs only because Aroclor is an undefined mixture of several constituents. Pp-wt strain was able to deplete 4CI-PCB almost by 90%, whereas it was able to deplete 2CI-PCB by almost 30% (Fig. 4). Higher depletion rate of 4CI-PCB paralleled the higher growth of Pp-wt in this PCB compared with that of 2CI-PCB. A gnotobiotic system (Simons, 1996) was set up, which consisted of four canonical plant-microbe combinations, all grown in the presence of $53 \mu\text{M}$ 2CI-PCB. Sand was used instead of soil because PCBs are known to bind irre-

versibly to soil particles, thereby reducing the bio-availability. We established that the plant genotypes (data not shown) as well as the microbial strains (Fig. 4) are not adversely affected in growth at $53 \mu\text{M}$ PCB concentration used in these studies. Bacterial counts and PCB depletion studies were done (a) using sand adhering to the roots, and (b) using the remaining sand in the tube (Fig. 5). Uninoculated controls showed 98.6% and 98.1% of PCBs remaining at 7 and 14 d, respectively. This indicated negligible natural degradation of PCBs during the course of the experiment. Microbial populations showed growth between 1 and 2 \log_{10} units in the four combinations of

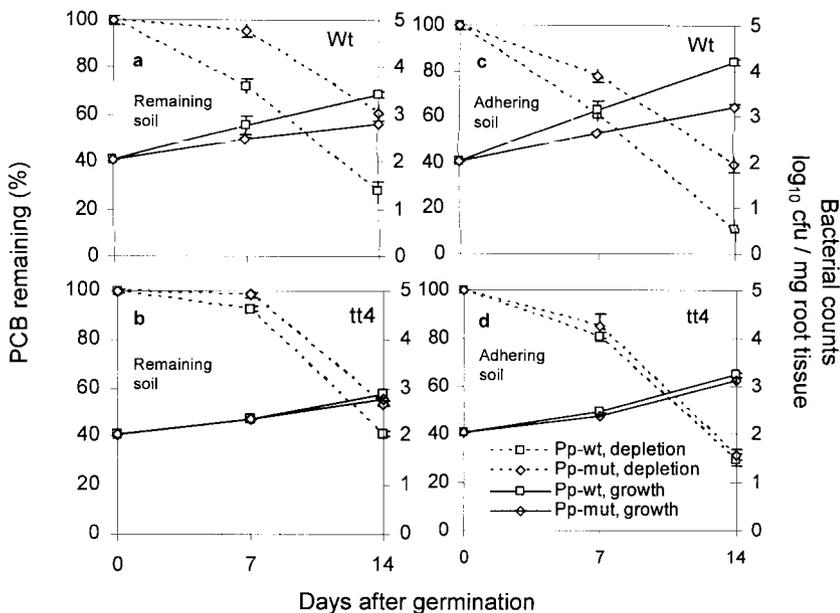


Figure 5. PCB removal by the phenylpropanoid-utilizing Pp-wt and Pp-mut strains colonizing roots of flavonoid-producing wild-type (a and c) or its null mutant *tt4* (b and d) plants. A gnotobiotic system was setup as previously described (Simons et al., 1996) using acid-washed sand in 200-mL glass test tubes. 2CI-PCB was added at $53 \mu\text{M}$ level per test tube. Inoculation of seeds with individual bacterial strains and bacterial counts in the adhering soil (c and d) were done as described in Figure 2 legend. Dilution plating was carried out with the remaining soil in the test tube (a and b) to determine the bacterial counts. PCB removal was quantified as described in Figure 4 legend. Replicates and experiments were set up as described in Figure 2 legend. Vertical bars represent SEs. Experiments were repeated at least three times.

inoculations studied. As expected, microbial populations were higher near the plant roots (Fig. 5c). In all cases studied, significant amounts of PCB depletion were observed. The least amount of PCB depletion was seen in the case of the flavonoid null mutant *tt4* supporting the growth of either of the two *Pseudomonas* spp. strains (Fig. 5b), which is expected based on results from the colonization studies. In the total soil, an average depletion of almost 50% of PCB was observed after the microbial populations increased by 1 log₁₀ unit. Hence, a small increase in microbial population could stimulate a significant amount of PCB depletion. Near the roots (Fig. 5c), the same plant genotype supported a higher increase of 2.1 log₁₀ units in the two microbial populations. Concomitantly, there was almost a 70% depletion of the PCB. The most remarkable depletion was achieved by Pp-wt colonizing the wild-type Arabidopsis plant roots. In the region surrounding these roots, more than 90% of PCB was removed within 2 weeks. In the same period, microbial population increased by 2 log₁₀ units. Pp-mut strain shows a slightly higher growth and PCB-depletion when adhering to the roots of wild-type plants as compared with those of *tt4* plants. This could perhaps be due to altered metabolism in both plants and microbes used in such a way as to provide a slight nutritional advantage to Pp-mut strain on the roots of wild-type plants.

In summary, a small increase in population (10- to 100-fold) could lead to a significant depletion of PCB present in the system. Pp-wt strain degraded 90% of PCBs on wild-type plants as compared with only 60% by the Pp-mut strain. Rhizoengineering-based enhancement of PCB depletion is, therefore, clearly evident in the gnotobiotic system used in our studies. Wider application of this rhizoengineering approach is not limited by the strain used in the study. Microbes that process such dual properties of degradation of natural product and pollutants by soil microbes have been previously reported (Donnelly, 1994) and can be directly used for rhizoengineering purposes. The approach shown here can be applied to enhance root colonization of those microbes, which may have additional pollutant degradation properties such as polyaromatic hydrocarbons (Kuiper, 2001). It is tempting to speculate that under field conditions, the strains targeted for rhizoengineering may face competition by some resident microbes capable of phenylpropanoid utilization. However, such competition is likely to be offset by the number of introduced microbes to be used in rhizoengineering. In addition, compared with Arabidopsis used in these studies, which has small root biomass and correspondingly lower exudate levels, plants in the field situation with higher root biomass are likely to have higher amounts of exudates leading to higher microbial colonization levels and thus to improved removal of pollutants. The strategy of enhancing the soil microbial populations using natural secondary

metabolites exuded by wild-type plants could therefore be applied to the removal of many classes of pollutants in vegetated soils. In cases where the pollutant-degrading microbes are not known to use secondary metabolites (Donnelly, 1994), such property could be introduced into them using genetic-engineering methods. This strategy is also likely to be applicable for improving competitive abilities of bio-control and biofertilization strains.

MATERIALS AND METHODS

Bacteria and Plants

Seeds of Arabidopsis plants were obtained from the Nottingham Arabidopsis Stock Center (UK). The *Pseudomonas putida* (Pillai and Swarup, 2002) PML2 strain (Pp-wt) is capable of using phenylpropanoids such as flavonoids (Pillai and Swarup, 2002), lignins, sinapates, coumarins, and indole compounds (this study), whereas *Flav1-1* (Pp-mut) is an auxotrophic mutant of PML2, which cannot use any of the above phenylpropanoids as a sole carbon source (Pillai and Swarup, 2002).

Rhizosphere Metabolomics

Surface-sterilized Arabidopsis seeds (300) were germinated on 0.8% (w/v) water agar plates that were kept in an inverted position to allow aeroponic-like growth of the roots. Root exudates were harvested by submerging the roots of 20-d-old plants in 2 mL of water for 1 h. RP-HPLC and ESI/MS were previously described (Pillai and Swarup, 2002). Quantitative analysis of the quercetin aglycone was carried out using SIM mode of the deprotonated molecule peak for acid-hydrolyzed root exudates. Intensity values obtained from the ESI/MS analysis for the individual compounds were plotted using function of the Mat Lab statistical package. The phenolic compounds in the root exudates were concentrated using disposable 3-mL LC-18 SPE tubes (Supelco Inc, Bellefonte, PA). Rhizosphere metabolomics are presented in Figure 1c in the same scale for wild type and the three mutants, where position on the horizontal axis represents different secondary metabolites, as listed in the supplementary information.

Bacterization, Root Colonization, and Competition Assays

Bacterization was carried out by treating 50 surface-sterilized seeds per milliliter of resuspended bacteria (8×10^4 colony forming units mL⁻¹) for 15 to 20 min at 28°C, which led to an establishment of 100 colony forming units per strain for each bacterial strain per seed. For competition studies, cells of Pp-wt and Pp-mut strains were mixed in equal proportions. Plants were grown in Magenta GA-7 boxes in a growth chamber maintained at 25°C and 70% relative humidity with 16 h of daylight. Fifty bacterized seeds were planted in 45 g of sterile potting mix (peat:vermiculate:acid washed sand, 1:1:1) in Magenta GA-7 boxes consisting of one replicate, whereas five such replicates were used for each treatment. Samples consisting of eight plants per replication were taken under axenic conditions at weekly intervals after sowing. Loosely adherent soil from roots was gently removed, and roots were carefully separated from the plants and macerated in 0.5 mL of 0.9% (w/v) NaCl. Viable counts of the inoculated strains were determined by dilution plating on Luria-Bertani agar plates supplemented with appropriate antibiotics. For each data point, means were calculated after log₁₀ transformation of the total bacterial count and the experiments were repeated three times.

Confocal Microscopy

Wild-type *Pseudomonas* spp. strain was tagged with a highly stable, broad host range vector pSMC2 that expresses *gfp* (Bloemberg, 1997). The growth characteristics of the *gfp*-tagged Pp-wt (Pp-wt/*gfp*) were identical to that of the parental Pp-wt strain. Secondary roots were viewed for the presence of Pp-wt/*gfp* cells using a laser scanning confocal microscope (LSM 410, Zeiss,

Welwyn Garden City, UK) with an excitation 488-nm wavelength and detection of the emission at 520 nm, respectively.

PCB Depletion Studies

Three PCB compounds, 2Cl-biphenyl, 4Cl-biphenyl (Riedel-de Haen, Germany), and Aroclor 1254 (Supelco, Bellefonte, PA), in 100 mg mL⁻¹ acetone were used at 53 μM final concentrations in liquid cultures or in a gnotobiotic system setup according to Simons et al. (1996) with minor modifications. Aroclor depletion was not quantified because it is an undefined mixture of PCBs. Acid-washed sand (10 g; GPR, BDH Laboratory Supplies, Poole, UK) was used in glass test tubes because PCBs are known to bind irreversibly/strongly to both soil and plastic. Fifteen to 20 seeds were sown per test tube, and five such replicates were setup per treatment for each time point. The experiments were repeated three times.

PCB Extraction and GC Analysis

PCBs from soil samples (Mohn, 1997) and cultures (Mackova, 1997) were extracted. Sample analysis (Gill, 1996) was carried out using GC-MS (QP5050 gas chromatographer-mass spectrometer, Shimadzu, Tokyo) equipped with DB-5 fused silica capillary column (30-m × 0.32-mm i.d.; film thickness, 0.25 μm; J&W Scientific, Folsom, CA). Standards and samples were analyzed in SIM mode for quantitative analysis. Total PCB removal was calculated using three PCB compounds as calibration standards.

ACKNOWLEDGMENTS

We thank the Protein and Proteomics Centre (Department of Biological Sciences, National University of Singapore) for HPLC and ESI/MS. The assistance of S. Bhinu (Department of Biological Sciences, National University of Singapore) for sharing his auxotrophic mutant collection is gratefully acknowledged.

Received October 16, 2002; returned for revision January 27, 2003; accepted January 31, 2003.

LITERATURE CITED

- Agency for Toxic Substances and Disease Registry** (2001) Top 20 hazardous substances from the CERCLA priority list of hazardous substances for 2001. <http://www.atsdr.cdc.gov/cxc3.html>
- Bloemberg GV, O'Toole GA, Lugtenberg BJJ, Kolter R** (1997) Green fluorescent protein as a marker for *Pseudomonas* spp. *Appl Environ Microbiol* **63**: 4543–4551
- Brimecombe MJ, De Leij FA, Lynch JM** (2001) The effect of root exudates on rhizosphere microbial populations. In R Pinton, Z Varanini, P Nannipieri, eds, *The Rhizosphere*. Marcel Dekker, New York, pp 95–140
- Dekkers LC, Mulders IHM, Phoelich CC, Woeng TFCCA, Wijffjes AHM, Lugtenberg BJJ** (2000) The *sss* colonization gene of the tomato-*Fusarium oxysporum* f. sp. *radicis-lycopersici* biocontrol strain *Pseudomonas fluorescens* WCS365 can improve root colonization of other wild type *Pseudomonas* bacteria. *Mol Plant-Microbe Interact* **13**: 1177–1183
- Donnelly KP, Hegde SR, Fletcher SH** (1994) Growth of PCB-degrading bacteria on compounds from photosynthetic plants. *Chemosphere* **28**: 981–988
- Gill US, Schwartz HM, Wheatley B** (1996) Development of a method for analysis of PCBs congeners and organochlorine pesticides in blood/serum. *Chemosphere* **32**: 1055–1061
- Kuiper I, Bloemberg GV, Lugtenberg BJJ** (2001) Selection of a plant-bacterium pair as a novel tool for rhizostimulation of polycyclic aromatic hydrocarbon-degrading bacteria. *Mol Plant-Microbe Interact* **14**: 1197–1205
- Lugtenberg BJJ, Dekkers LL, Bloemberg GV** (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annu Rev Phytopathol* **39**: 461–490
- Mackova M, Macek T, Ocenaskova J, Burkhard J, Demnerova K, Pazlarova J** (1997) Biodegradation of polychlorinated biphenyls by plant cells. *Int Biodeterior Biodegrad* **39**: 317–325
- Metting FB Jr** (1992) Structure and physiological ecology of soil microbial communities. In FB Metting Jr, ed, *Soil Microbial Ecology*. Marcel Dekker, New York, pp 3–25
- Mohn WW, Westenberg K, Cullen WR, Reimer KJ** (1997) Aerobic biodegradation of biphenyl and polychlorinated biphenyls by Arctic soil microorganisms. *Appl Environ Microbiol* **63**: 3378–3384
- National Environment Indicator Series** (1996) SOE Technical Supplement No. 98-1. http://www.ec.gc.ca/Ind/english/Toxic/Tech_Sup/txsup8_e.cfm
- Nesi N, Debeaujon I, Jond C, Pelletier G, Caboche M, Lepiniec L** (2000) The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of DFR and BAN genes in *Arabidopsis* siliques. *Plant Cell* **12**: 1863–1878
- Normander B, Hendriksen NB, Nybroe O** (1999) Green fluorescent protein-marked *Pseudomonas fluorescens*: localization, viability, and activity in the natural barley rhizosphere. *Appl Environ Microbiol* **65**: 4646–4651
- O'Connell KP, Goodman RM, Handelsman J** (1996) Engineering the rhizosphere: expressing a bias. *Trends Biotechnol* **14**: 83–88
- Oger P, Petit A, Dessaux Y** (1997) Genetically engineered plants producing opines alter their biological environment. *Nat Biotechnol* **15**: 369–372
- Pelletier MK, Burbulis IE, Shirley BW** (1999) Disruption of specific flavonoid genes enhances the accumulation of flavonoid enzymes and products in *Arabidopsis* seedlings. *Plant Mol Biol* **40**: 45–54
- Pillai BVS, Swarup S** (2002) Elucidation of the flavonoid catabolism pathway in *Pseudomonas putida* PML2 by comparative metabolic profiling. *Appl Environ Microbiol* **68**: 143–151
- Savka MA, Farrand SK** (1997) Modification of rhizobacterial populations by engineering bacterium utilization of novel plant-produced resource. *Nat Biotechnol* **15**: 363–368
- Simons M, van der Bijl AJ, Brand I, de Wager LE, Wijffelman CA, Lugtenberg BJJ** (1996) Gnotobiotic system for studying rhizosphere colonization by plant growth promoting *Pseudomonas* bacteria. *Mol Plant-Microbe Interact* **9**: 600–607
- Walker AR, Davison PA, Bolognesi-Winfield AC, James CM, Srinivasan N, Blundell TL, Esch JJ, Marks MD, Gray JC** (1999) The TRANSPARENT TESTA GLABRA1 locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. *Plant Cell* **11**: 1337–1349
- Whipps JM** (1990) Carbon economy. In JM Lynch, ed, *The Rhizosphere*. Wiley, New York, pp 59–97