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Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants

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Phytoremediation of highly water soluble and volatile organic xenobiotics is often inefficient because plants do not completely degrade these compounds through their rhizospheres. This results in phytotoxicity and/or volatilization of chemicals through the leaves, which can cause additional environmental problems. We demonstrate that endophytic bacteria equipped with the appropriate degradation pathway improve the *in planta* degradation of toluene. We introduced the pTOM toluene-degradation plasmid of *Burkholderia cepacia* G4 into *B. cepacia* L.S.2.4, a natural endophyte of yellow lupine. After surface-sterilized lupine seeds were successfully inoculated with the recombinant strain, the engineered endophytic bacteria strongly degraded toluene, resulting in a marked decrease in its phytotoxicity, and a 50–70% reduction of its evapotranspiration through the leaves. This strategy promises to improve the efficiency of phytoremediating volatile organic contaminants.

Emerging phytoremediation technologies have been applied at various scales to treat moderately hydrophobic pollutants, such as benzene, toluene, ethylbenzene and xylene (BTEX) compounds, chlorinated solvents, nitrotoluene ammunition wastes and excess nutrients[1]. Phytoremediation of organic xenobiotics is based on interactions between plants and their associated microorganisms in a process whereby plants draw pollutants, including polyaromatic hydrocarbons (PAHs), into their rhizosphere via the transpiration stream[2]; subsequently, microorganism-mediated degradation occurs in the plant itself, in the rhizosphere or in both.

The fate of the contaminant in the rhizosphere-root system largely depends on its physicochemical properties. Organic xenobiotics with a log K_{ow} (octanol/water partition coefficient) < 1 are considered to be very water-soluble, and plant roots do not generally accumulate them at a rate surpassing passive influx into the transpiration stream[3]. Contaminants with a log K_{ow} > 3.5 show high sorption to the roots, but slow or no translocation to the stems and leaves[4]. However, plants readily take up organic xenobiotics with a log K_{ow} between 0.5 and 3.5, as well as weak electrolytes (weak acids and bases or amphoteres as herbicides). These compounds seem to enter the xylem faster than the soil and rhizosphere microflora can degrade them, even if the latter is enriched with degrading bacteria[5]. Once taken up, plants metabolize these contaminants, although some of them, or their metabolites, such as trichloroethene (TCE), which is transformed into trichloro acetic acid, can be toxic[6]. Alternatively, plants preferentially release volatile pollutants, such as BTEX compounds and TCE and their metabolites, into the environment by evaporation via the leaves, which calls into question the merits of phytoremediation[7–10]. There is thus a need for different strategies to improve the phytoremediation of such contaminants.

In this study we aimed to increase the degradation of volatile, water-soluble organic contaminants during their transport in the plant's vascular system using engineered endophytic bacteria. Endophytic bacteria reside within the living tissue of a plant without substantively harming it[11,12]. They are ubiquitous in most plant species, residing latently or actively colonizing the tissues. Bacterial endophytes are highly diverse with many different taxa colonizing a wide variety of plant species[13]. It has been reported[14] that substantial numbers of endophytes (10^3–10^6 cells) can colonize the vascular system (phloem, xylem). The highest densities usually are observed in the roots and decrease progressively from the stem to the leaves.

In this study we demonstrate that a genetically modified endophytic strain of *B. cepacia*, whose host plant is the yellow lupine (*Lupinus luteus L.*), improves *in planta* degradation and reduces evapotranspiration of toluene, a moderately hydrophobic (log K_{ow} = 2.69 at 20 °C) volatile compound.

RESULTS

Inoculation of yellow lupine with *B. cepacia*

We chose yellow lupine as our plant model. *B. cepacia* BU0072 (refs. 15,16), which is derived from the endophytic strain *B. cepacia* L.S.2.4, and its toluene-degrading derivative VM1330, were used to inoculate

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Table 1 Growth characteristics and number of bacterial colonies isolated from roots and shoots of *L. luteus* plants

Inoculum[a]	Plant part	284+gluc[b]	284+Ni+Km+gluc[b]	284+Ni+Km+tol[b]	284+tol[b]
No	Shoot	2.3×10^2 (2)[c]	0	0	0
No	Root	1.7×10^3 (3)	0	0	0
VM1330	Shoot	6.9×10^3 (1)	3.8×10^2 (1)	5.8×10^2 (1)	4.3×10^2 (1)
VM1330	Root	9.5×10^3 (1)	2.2×10^2 (1)	1.7×10^2 (1)	1.9×10^2 (1)
BU0072	Shoot	1.3×10^4 (2)	2.2×10^2 (1)	0	0
BU0072	Root	1.5×10^3 (3)	1.5×10^3 (1)	0	0
G4	Shoot	5.7×10^4 (2)	0	0	1.0×10^4 (1)
G4	Root	7.8×10^4 (2)	0	0	1.5×10^2 (1)

[a]*L. luteus* plants were inoculated with *B. cepacia* strains VM1330, BU0072 and G4. As controls, plants without inoculum were analyzed. [b]Ni: 1 mM nickel; Km: 100 μg/ml kanamycin; tol: toluene vapor as C-source; gluc: gluconate as C-source. [c]The number of bacteria was determined 21 d after inoculation. The number of bacteria is expressed per g fresh weight. Data are the average of three experiments. Numbers in parentheses are the numbers of different morphological types of bacteria as observed visually.

the plant. Both are resistant to kanamycin and nickel. In addition, we tested the endophytic characteristics of the toluene-degrading soil bacterium *B. cepacia* G4.

Twenty-one days after inoculating the bacteria, the plants were harvested; roots and shoots were separated, surface-sterilized, rinsed and macerated. The total numbers of specific bacteria in the crushed materials, as well as their specific growth characteristics (resistance properties and toluene degradation) were determined on different selective media (**Table 1**). The number of CFUs (colony forming units) was calculated per gram of fresh weight of roots or shoots. We found that three weeks after inoculation, all three *B. cepacia* strains could be isolated from yellow lupine. For control plants without inoculum, no bacteria were found on the selective media, except on nonselective medium (284 + glucose), demonstrating that despite surface sterilization of the seeds, endogenous endophytic bacteria remained in the plants.

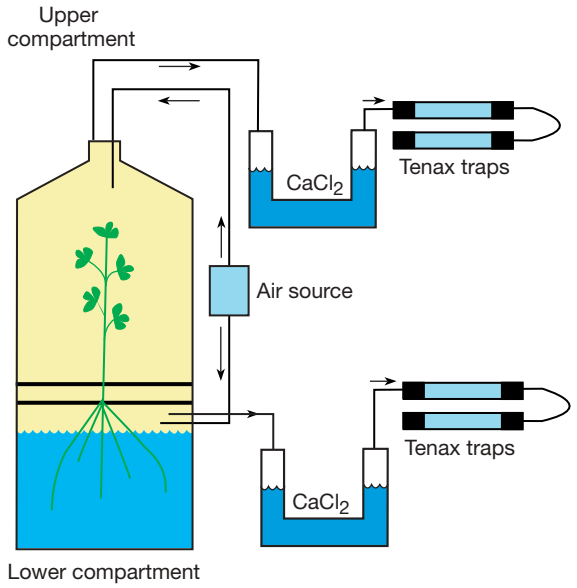

Figure 1 Schematic representation of the experimental setup for measuring toluene evapotranspiration. Yellow lupine plants are grown as hydroponics in a two-compartment glass cuvette system. Continuous airflow allows toluene from the gas phases from both compartments to collect on Tenax traps. A $CaCl_2$-filled column was installed to prevent condensation of water in the Tenax traps. Tenax traps were cooled with dry ice to optimize adsorption capacities.

As expected, bacteria isolated from the shoots and roots of yellow lupine inoculated with VM1330 (Km^R, Ni^R, Tol^+) grew on all three selective media. Plants inoculated with BU0072 (Km^R, Ni^R, Tol^-) harbored no toluene-degrading bacteria. From plants inoculated with strain G4 (Tol^+), isolated bacteria grew on a medium containing toluene, but only in the absence of nickel and kanamycin.

Using Repetitive Extragenic Palindromic (REP)-PCR we demonstrated that the bacteria isolated on the selective media had the same genetic fingerprints as *B. cepacia* G4, BU0072 and VM1330, respectively (results not shown). In addition, the presence of *nre* (strain BU0072), pTOM (strain G4), and both *nre* and pTOM (strain VM1330) was determined by PCR, confirming that the three strains had colonized the plants. Bacteria isolated from the control plants showed a different REP-PCR pattern from strains BU0072, G4 and VM1330. These endophytic bacteria, which were also found after inoculation with *B. cepacia* BU0072 and G4, were further characterized and found to belong to typical endophytic and rhizosphere-colonizing species. Based on their REP-PCR patterns, five distinct species were found and these were identified by the sequence of their 16S rDNA: (i) *Paenibacillus* sp., a typical plant-associated, nitrogen-fixing bacterium of the rhizosphere, which has been found as an endophyte in plants as diverse as pine and potato[17,18]; (ii) *Bacillus megaterium*, (iii) *Pantoea* sp. and (iv) a *Pseudomonas* sp., which all have been reported to be endophytic bacteria in pea[19], a member of the Fabaceae, like yellow lupine; and (v) a *Bacillus novalis* sp. nov., which was recently isolated from the Drentse A grasslands[20]. None of these bacteria was able to grow on toluene as a carbon source.

Selective enrichment of endophytic bacteria by toluene

We investigated how the presence and concentration of toluene affected colonization by the bacteria. Earlier results had indicated that growing plants on contaminated soil could selectively enhance the prevalence of endophytes containing the degradation pathways for specific pollutants[21]. After 21 d of growth without toluene, the plants were transferred to glass cuvettes containing toluene in the lower compartment at final concentrations of 0, 100, 500 or 1,000 mg/l. After 96 h the plants were harvested, and their shoots and roots examined for endophytic colonization. Samples and dilutions of 100 μl were plated on different media. We could not discern a clear effect of the toluene concentration on the efficiency of colonization by different endophytic strains, and therefore, differences in the toluene-degradation properties of the inoculated plants will not reflect differences in the density of the endophytic population. An average of 10^5–10^6 CFU per gram fresh weight was found, and as in the experiment described in **Table 1**, growth on selective media was observed only after plants had been inoculated with the appropriate strains. Strain G4 was, in general, the most efficient colonizer of yellow lupine (10^6 CFU per gram fresh weight compared to 10^5 CFU for BU0072 and VM1330). In addition, the same endogenous endophytic bacteria were found in the control plants after surface sterilization.

Effect on plant growth during hydroponic cultivation

We examined the effect of toluene on the hydroponic growth of plants inoculated with *B. cepacia* VM1330, BU0072 or G4 and compared it with the effect on noninoculated controls. The experiments were car-

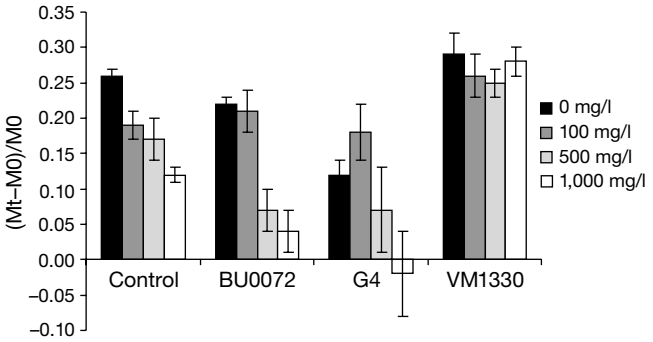

Figure 2 Difference in biomass (g) between inoculated and control yellow lupine plants, before and after adding toluene. Plants were grown as hydroponics in the glass cuvette system described in **Figure 1**. Noninoculated control plants and lupine plants inoculated with *B. cepacia* strains VM1330, BU0072 and G4 were used. M0, plant weight (g) before addition of toluene; Mt, plant weight (g) 96 h after toluene addition. The concentrations of toluene were 0, 100, 500 and 1,000 mg/l, respectively. Standard deviations are indicated as bars. The statistical significance of the results was confirmed at the 5% level using a two-way ANOVA model, separately exploring treatment (bacterial inocula) and toluene doses.

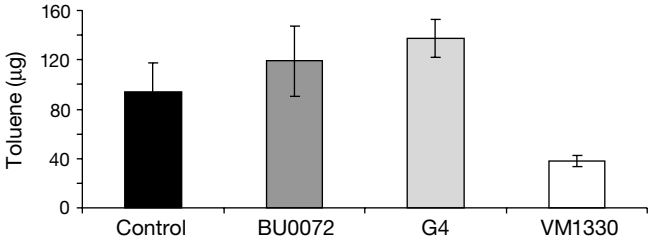

Figure 3 Total amount of toluene (µg) detected in Tenax traps connected with the upper compartment (containing the aerial part of *L. luteus* plant) determined by GC-MS. For this experiment, noninoculated control plants and lupine plants inoculated with *B. cepacia* strains VM1330, BU0072 and G4 were used. The statistical significance of the results was confirmed at the 5% level using a one-way ANOVA model.

ried out in the glass cuvette system schematically presented in **Figure 1**. The growth indices were calculated as the difference in plant's fresh weight between the onset of the experiment and after 96 h exposure to different concentrations of toluene. In the absence of toluene, plants inoculated with *B. cepacia* G4 produced significantly ($P = 0.05$) less biomass than the control plants and those inoculated with *B. cepacia* BU0072 and VM1330 (**Fig. 2**). This indicates that high numbers of the environmental *B. cepacia* G4 strain, which is not known to be a natural endophyte of yellow lupine, has a negative effect on plant development.

For plants and bacteria incubated in the presence of toluene, the growth indices suggested that increasing levels of toluene resulted in greater phytotoxicity (**Fig. 2**). However, plants inoculated with the endophytic strain *B. cepacia* VM1330, which can efficiently metabolize toluene, showed no difference in growth compared to controls without toluene. Even at concentrations of 1,000 mg/l toluene in the growth medium, these plants showed no signs of phytotoxicity, in contrast to the control plants that experienced phytotoxicity at levels above 100 mg/l. This shows that strain VM1330 efficiently assists its host plant in overcoming toluene's phytotoxicity. Plants inoculated with *B. cepacia* BU0072 and G4 show phytotoxicity to toluene that is similar to that of the noninoculated control plants, suggesting that the combination of natural endophytic behavior plus the presence of the toluene degradation capacity is required to protect the plant. The statistical significance of the results was confirmed at the 5% level using a two-way ANOVA model, separately exploring treatment (bacterial inocula) and toluene doses.

Toluene degradation and evapotranspiration

After adding toluene at a sub-phytotoxic concentration of 100 mg/l, we measured the amount of toluene that is evapotranspired through the aerial parts of the plant (upper compartment) as well as its disappearance from Hoagland's nutrient solution (lower compartment) using gas chromatography/mass spectrometry (GC-MS) (**Fig. 3**).

Compared to control plants and plants inoculated with *B. cepacia* BU0072 or G4, those inoculated with *B. cepacia* VM1330 released 50–70% less toluene in the upper compartment (**Fig. 3**). This result shows that this toluene-degrading endophytic strain not only protects its host plant against phytotoxicity, but also significantly ($P = 0.05$) lowers toluene evapotranspiration through the aerial parts, even at levels that are not toxic to control plants. No significant ($P = 0.05$) differences in the concentrations of evapotranspired toluene were observed between plants inoculated with BU0072 or G4, and the noninoculated controls. The statistical significance of the reduced toluene release in the presence of strain VM1330 was confirmed at the 5% level using a one-way ANOVA model.

To further examine the fate of the toluene, we also analyzed the amount that evaporated from the Hoagland's solution in the gas-phase of the lower compartment. The smallest amount of evaporated toluene, 2,523 (±853) µg, was obtained from plants inoculated with *B. cepacia* VM1330, compared to 3,378 (±987) µg, 4,362 (±733) µg and 7,367 (±298) µg for the control plants and the plants inoculated with BU0072 or G4, respectively. These results show that together the endophytic strain *B. cepacia* VM1330 and its host plant, yellow lupine, improve the degradation of toluene, lowering both its phytotoxicity and release by evapotranspiration.

Effect on plant growth during greenhouse studies

We further examined the protective effect of the endophytic strain *B. cepacia* VM1330 on its host plant in greenhouse studies growing the plants on a nonsterile sandy soil. It is clear that control plants suffer from phytotoxic effects when irrigated for two weeks with water that contains toluene at a concentration of 100 mg/l, and that they cannot survive higher toluene concentrations (**Figs. 4** and **5**). Inoculation of yellow lupine with *B. cepacia* BU0072 had a slightly positive effect, as it reduced toluene phytotoxicity in the group treated with 100 mg/l. Plants exposed to the higher toluene concentrations died within one week. Inoculation with the toluene-degrading *B. cepacia* G4 strain resulted in a partial reduction of toluene phytotoxicity, which can be explained by degradation of toluene in the rhizosphere. A clear protective effect of *B. cepacia* VM1330 was observed on the growth of yellow lupine at all toluene concentrations tested, including the 500 mg/l doses. The statistical significance of the results was confirmed at the 5% level using a two-way ANOVA model, separately exploring treatment (bacterial inocula) and toluene doses. These results confirm our hypothesis that endophytic bacteria, when equipped with the appropriate degradation pathway, can help plants survive under conditions of elevated levels of toluene.

DISCUSSION

In this paper we describe how an engineered endophytic bacterium can improve the phytoremediation of an organic contaminant. We

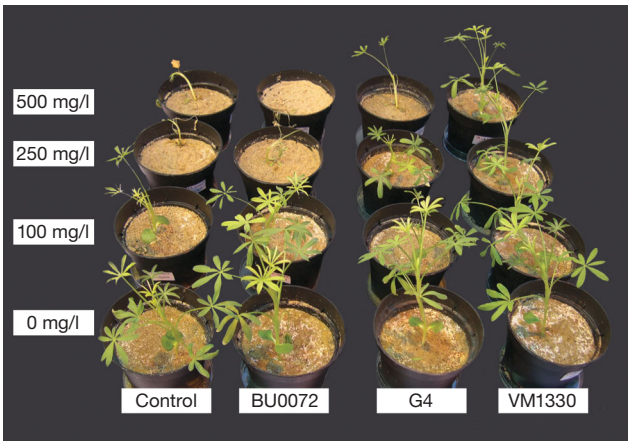

Figure 4 Phytotoxic effect of toluene on yellow lupine grown in nonsterile sandy soil under the greenhouse conditions. The labels indicate the control plants and lupine plants inoculated with *B. cepacia* strains VM1330, BU0072 and G4. Plants were irrigated every other day with half-strength Hoagland's solution to which toluene was added at concentrations of 0, 100, 250 and 500 mg/l. A picture of representative plants was taken after 14 d of irrigation with toluene-containing solutions.

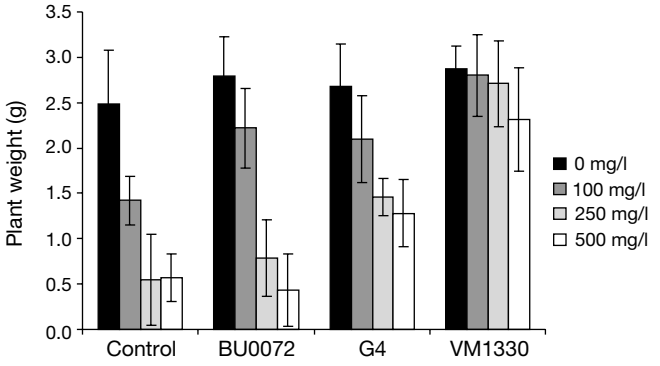

Figure 5 Biomass (g) of yellow lupine plants, grown in nonsterile sandy soil under the greenhouse conditions, after 14-d exposure to the different toluene concentrations. For this experiment, noninoculated control plants and lupine plants inoculated with *B. cepacia* strains VM1330, BU0072 and G4 were used. Plants were irrigated every other day with half-strength Hoagland's solution to which toluene was added at concentrations of 0, 100, 250 and 500 mg/l. Standard deviations are indicated as bars. The statistical significance of the results was confirmed at the 5% level using a two-way ANOVA model, separately exploring treatment (bacterial inocula) and toluene doses.

demonstrate that an endophytic bacterium equipped with the appropriate degradation pathway not only protects its host plant against the phytotoxic effect of an environmental contaminant, but also improves the overall degradation of the contaminant, resulting in its decreased evapotranspiration to the environment.

The protective effect of the endophytic bacteria was demonstrated for plants grown either as hydroponics or in nonsterile soil under greenhouse conditions. The results also show that the presence of *B. cepacia* VM1330, a toluene-degrading endophyte, provides much better protection against the phytotoxic effects of toluene than that obtained when the rhizosphere of the plant is inoculated with *B. cepacia* G4, a toluene-degrading soil bacterium (**Figs. 4** and **5**).

The introduction of the pTOM plasmid into *B. cepacia* BU0072, which resulted in strain *B. cepacia* VM1330, was done through natural gene transfer (conjugation). Although for practical purposes we used a marked recipient strain, which allows us to monitor the colonization of yellow lupine, this experiment can easily be repeated using a naturally-occurring endophytic recipient, such as *B. cepacia* L.S.2.4, and an autotrophic mutant of *B. cepacia* G4. Accordingly, the transconjugants obtained by conjugation using a naturally occurring endophytic recipient should not be considered genetically modified microorganisms (GMO), a factor that may facilitate their application and public acceptance. It will be relatively straightforward to construct, by natural gene transfer, a collection of endophytic bacteria with *a la carte* degradation properties because many endophytic bacteria are closely related to environmental strains that carry degradation pathways for a broad spectrum of organic xenobiotics on mobile DNA elements. For example, we are constructing derivatives of *B. cepacia* L.S.2.4 that constitutively express the *tom*A toluene-ortho-monooxygenase of pTOM[22,23]. This strain will be applied to improve the phytoremediation-based containment of TCE-contaminated groundwater plumes. Other applications we envisage include the use of engineered endophytic bacteria to degrade pesticide and herbicide residues in crop plants, addressing important food safety related issues.

The next step will be to expand and apply the work at various levels to poplar (*Populus* sp.) and willow (*Salix* sp.) to phytoremediate groundwater contaminated with water-soluble organics. A collection of approximately 150 different endophytic bacteria from poplar and willow is available, and these strains have been identified and characterized (F. Porteous Moore, T.B., B.B., L.O., C.D. Campbell, J.V., D.v.d.L. and E.R.B. Moore, unpublished data). We have also shown the inoculation of poplar with a GFP-marked endophytic *Pseudomonas* sp., demonstrating the generalizability of the approach[24]. We can reasonably hypothesize that endophytic bacteria, possessing the genetic information required to efficiently degrade an organic contaminant, promote its breakdown as it moves through the plant's vascular system. In trees, the time between the uptake of the pollutant by the roots and its arrival in the leaves can take several hours to days[25], allowing sufficient time for efficient degradation by endophytic bacteria in the xylem.

We expect that the application of genetically engineered endophytic bacteria will become a general strategy to improve the efficiency of phytoremediating volatile organic contaminants and other water soluble organic xenobiotics, and that the technique will gain regulatory and public acceptance.

METHODS

Construction of a toluene-degrading endophyte. *B. cepacia* strain BU0072 (Ni^R, Km^R), a derivative of the endophytic bacterium *B. cepacia* L.S.2.4, was used[15,16]. *B. cepacia* G4 (pTOM, Tol^+)[26] served as a donor strain for toluene degradation. After conjugation[16], transconjugants that were resistant to nickel plus kanamycin and could grow on toluene as sole carbon source were selected on '284' minimal medium[27] supplemented with 1 mM $NiCl_2$ plus 100 µg/ml kanamycin, while the plates were incubated under a toluene atmosphere to provide the carbon source. The 284 medium contains per liter distilled water: 6.06g Tris-HCl, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g $NaSO_4$, 0.20 g $MgCl_2 \cdot 6H_2O$, 0.03 g $CaCl_2 \cdot 2\ H_2O$, 40 mg $Na_2HPO_4 \cdot 2\ H_2O$, 10 ml Fe(III)NH_4 citrate solution (containing 48 mg/100 ml), 1 ml microelements solution, final pH 7. A carbon source, such as gluconate, is added at 0.2% (wt/vol). The microelement solution contains per liter distilled water: 1.3 ml 25% HCl, 144 mg $ZnSO_4 \cdot 7\ H_2O$, 100 mg $MnCl_2 \cdot 4\ H_2O$, 62 mg H_3BO_3, 190 mg $CoCl_2 \cdot 6\ H_2O$, 17 mg $CuCl_2 \cdot 2\ H_2O$, 24 mg $NiCl_2 \cdot 6\ H_2O$ and 36 mg $NaMoO_4 \cdot 2\ H_2O$. The presence of the *nre* Ni resistance marker and the pTOM plasmid in the transconjugants

was confirmed with PCR using *nre* and pTOM specific primers, respectively. A representative transconjugant, *B. cepacia* strain VM1330, which had the correct genetic makeup and grew under the appropriate selective conditions with toluene as sole carbon source, was selected for further studies.

The stability of the pTOM plasmid in VM1330 was verified by growing the strain for 100 generations on nonselective 284 gluconate minimal medium in the absence of toluene. Then, 100 individual colonies were selected on nonselective medium, and subsequently replica-plated on 284 minimal medium supplemented with 1 mM $NiCl_2$ plus 100 μg/ml kanamycin, while the plates were incubated under a toluene atmosphere to provide the carbon source. All colonies tested thrived, demonstrating the stability of the toluene-degradation properties located on the pTOM plasmid (less than 1% loss of the plasmid after growth for 100 generations under nonselective conditions), as well as the other resistance markers.

Inoculation of yellow lupine with *B. cepacia*. *B. cepacia* VM1330 was grown in 284 gluconate medium (250 ml culture) at 22 °C on a rotary shaker for approximately 7 d until a density of 10^{+9} CFU/ml was reached (OD_{660} of 1). The cells were collected by centrifugation, washed twice in 10 mM $MgSO_4$ and suspended in 1/10 of the original volume 10 mM $MgSO_4$ to obtain an inoculum with a cell density of 10^{10} CFU/ml.

Seeds of *L. luteus L.* were surface-sterilized for 30 min at 20 °C in a solution containing 1% active chloride (wt/vol, added as a NaOCl solution) and 1 droplet Tween 80 per 100 ml solution. The seeds then were rinsed three times for 1 min in sterile water and dried on sterile filter paper. To test the efficiency of sterilization, the seeds were incubated for 3 d at 30 °C on 869 medium[28], which contains per liter distilled water: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 g D-glucose, 0.345 g $CaCl_2 \cdot 2\,H_2O$ (pH 7). Seeds were considered sterile when no bacterial growth was observed. Five surface sterile seeds of *L. luteus L.* were planted in a sterile plastic jar (800 ml), completely filled with sterilized perlite and saturated with 400 ml of a half-strength sterile Hoagland's nutrient solution. Subsequently, the bacterial inoculum was added to each jar at a final concentration of 10^8 CFU/ml Hoagland's solution (three Hoagland's stock solutions are prepared; solution 1: macroelements, containing 102 g KNO_3, 70.8 g $Ca(NO_3)_2 \cdot 4\,H_2O$, 23 g $NH_4H_2PO_4$, 49 g $MgSO_4 \cdot 7\,H_2O$ per 10 liters distilled water; solution 2: Fe solution containing 1.9 g $FeSO_4 \cdot 7\,H_2O$, 1.25 g EDTA-di-Na-salt per 250 ml distilled water; solution 3: microelements 2.86 g H_3BO_3, 1.81 g $MnCl_2 \cdot 4\,H_2O$, 0.08 g $CuSO_4 \cdot 5\,H_2O$, 0.09 g $H_2MoO_4 \cdot H_2O$, 0.22 g $ZnSO_4 \cdot 7\,H_2O$; Hoagland's solution contains per 10 liters: 1 liter solution 1, 6 ml solution 2 and 10 ml solution 3, pH 6.5). The jars were covered with sterile tinfoil to facilitate bacterial colonization and prevent contamination and dispersion of the inoculated bacteria through the air. After the seeds had germinated, perforations were made in the tinfoil and plants were allowed to grow through them over 21 d in a growth chamber (constant temperature of 22 °C, relative humidity 65%, and 14/10 h light and dark cycle, PAR (photosynthetic active radiation) 165 μmol/m^2s). The same procedure was used to inoculate *L. luteus L.* with the *B. cepacia* strains BU0072 and G4.

Recovery of endophytic bacteria. Plants were harvested after 21 d. Roots and shoots were treated separately. Fresh root and shoot material was vigorously washed in distilled water for 5 min, surface-sterilized for 5 min in a solution containing 1% active chloride (wt/vol, added as a NaOCl solution) supplemented with 1 droplet Tween 80 per 100 ml solution and rinsed three times in sterile distilled water. A 100 μl sample of the third rinsing water was plated on 869 medium to verify the efficiency of sterilization. After sterilization, the roots and shoots were macerated in 10 ml 10 mM $MgSO_4$ using a Polytron PT1200 mixer (Kinematica A6). Samples (100 μl) were plated on different selective and nonselective media to test for the presence of the endophytes and their characteristics.

Toluene degradation and phytotoxicity tests on hydroponics. Three-week-old *L. luteus L.* plants (both controls and those inoculated with *B. cepacia* VM1330, BU0072 or G4) were used to evaluate the phytotoxicity of toluene and its *in planta* degradation. The lupine plants were carefully taken out of the jars and their roots were vigorously rinsed in sterile water to remove bacteria from the surface. Subsequently, plants were grown hydroponically, settled in a two-compartment glass cuvette system (29 cm high; 9 cm in diameter) (**Fig. 1**). To avoid gas exchange between the upper and lower compartments, they were separated by a glass plate, with an insertion through which the stem of the plant was introduced. Each cuvette contained one plant and the space around the stem was made gas-tight with a Polyfilla exterior mixture (Polyfilla), so that shoots in the upper compartment and roots in the lower compartment were completely separated, allowing no gas exchange between them, except through the stem. The upper compartment, the glass plate and the lower compartment were sealed with Apiezon (Apiezon Products M&I Materials LTD). The lower compartment was filled with 300 ml of sterile, half-strength Hoagland's solution. Different toluene concentrations of 0, 100, 500, and 1,000 mg/l were added to the Hoagland's solution at the beginning of the experiment. The cuvettes with plants were placed in a growth chamber with constant temperature 22 °C and 14/10 hours light/dark cycle; photosynthetic active radiation 165 mmol/m^2s. Each compartment was connected with a synthetic air source (Air Liquide) with an inflow of 1 liter per hour. The phytotoxic effects of toluene under the different conditions were examined by determining the increase in plant biomass after 4 d.

We examined the effect of the different endophytic bacteria on toluene degradation and evapotranspiration using the following experimental setup. In the experiment where 100 mg/l toluene was added, two-serial linked Tenax traps (Capped Sample Tubes (Perkin Elmer) and Tenax 60/80 (Alltech)) were inserted in the outflow of each compartment to capture any transpired or volatilized toluene. The traps were changed regularly. A column filled with $CaCl_2$ was installed between the cuvettes and the Tenax traps as a trap to prevent condensation of water in the Tenax traps. To optimize the Tenax adsorption capabilities, the traps were cooled with dry ice. The whole experiment ran for 96 h, and toluene concentration in the traps was determined by GC-MS (Automatic thermal Desorption System ATD400, Auto System XL Gas Chromatograph, Turbo Mass Spectrometer, Perkin Elmer). All experiments were done in triplicate to allow statistical analysis of the data using ANOVA.

Toluene toxicity test under green house conditions. After 3 weeks of growth under the above conditions, control plants and lupine plants inoculated with *B. cepacia* strains VM1330, BU0072 and G4 were transferred into half-liter pots filled with a nonsterile sandy soil, irrigated with half-strength Hoagland's solution. Plants were allowed to stabilize for 3 d. Subsequently, plants were irrigated every other day with half-strength Hoagland's solution to which toluene was added at concentrations of 0, 100, 250 and 500 mg/l. After two weeks plants were harvested and their biomass was determined. For each treatment five replicas were done.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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