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ENDOPHYTIC BACTERIA FROM SEEDS OF NICOTIANA TABACUM CAN REDUCE CADMIUM PHYTOTOXICITY

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demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.
ENDOPHYTIC BACTERIA FROM SEEDS OF *NICOTIANA TABACUM* CAN REDUCE CADMIUM PHYTOTOXICITY

Chiara Mastretta,1 Safiyh Taghavi,2 Daniel van der Lelie,2 Alessio Mengoni,3 Francesca Galardi,4 Christina Gonnelli,4 Tanja Barac,1 Jana Boulet,1 Nele Weyens,1 and Jaco Vangronsveld1

1Environmental Biology, Hasselt University, Diepenbeek, Belgium
2Biology Department, Brookhaven National Laboratory, Upton, New York, USA
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Although endophytic bacteria seem to have a close association with their host plant, little is known about the influence of seed endophytic bacteria on initial plant development and on their interactions with plants under conditions of metal toxicity. In order to further elucidate this close relationship, we isolated endophytic bacteria from surface sterilized *Nicotiana tabacum* seeds that were collected from plants cultivated on a cadmium-(Cd) and zinc-enriched soil. Many of the isolated strains showed Cd tolerance. Sterilely grown tobacco plants were inoculated with either the endogenous microbial consortium, composed of cultivable and noncultivable strains; single strains; or defined consortia of the most representative cultivable strains. Subsequently, the effects of inoculation of endophytic bacteria on plant development and on metal and nutrient uptake were explored under conditions with and without exposure to Cd. In general, seed endophytes were found to have a positive effect on plant growth, as was illustrated by an increase in biomass production under conditions without Cd. In several cases, inoculation with endophytes resulted in improved biomass production under conditions of Cd stress, as well as in a higher plant Cd concentration and total plant Cd content compared to noninoculated plants. These results demonstrate the beneficial effects of seed endophytes on metal toxicity and accumulation, and suggest practical applications using inoculated seeds as a vector for plant beneficial bacteria.

KEY WORDS: endophytic bacteria, seed, metal toxicity, *Nicotiana tabacum*, cadmium (Cd), phytoextraction, nutrient balance

INTRODUCTION

Rhizobacteria are the most studied plant-associated bacteria and are often found to have beneficial effects on plant growth, *e.g.*, via the provision of essential elements, inhibition of colonization by pathogenic microorganisms, or by helping the plant to...
overcome stress responses to environmental insults (Hallmann et al., 1997). Similar beneficial effects have also been described for endophytic bacteria (for a review, see Mastretta et al., 2006). For their practical application, plant growth-promoting bacteria are frequently applied via seed coatings. Interestingly, not much information is available about plant beneficial endophytic bacteria isolated from seeds. Cultivable endophytic bacteria have been isolated from the seeds of coffee (Vega et al., 2005), Norway spruce (Cankar et al., 2005), rice (Tripathi et al., 2006), and rapeseed (Granér et al., 2003). However, not much is known about their ecological function. Some of these bacteria were found to have anti-fungal activity (Mukhopadhyay et al., 1996), but pathogenic bacteria were also found to inhabit the seeds (Schaad et al., 1995; Grum et al., 1998). For example, the infection of carrot seed by Xanthomonas campestris pv. carotae (Kuan, Minsavage, and Gabrielson, 1985) involves the bacterium gaining access to an internal part of the seed, for example the embryo, as was also reported for X. campestris pv. Manihotis (Elango and Lozano, 1980). Erwinia stewartii targets the endosperm (Rand and Cash, 1921), while X. campestris pv. malvacearum entered the seed coat (Brinkerhoff and Hunter, 1963). Barak, Koike, and Gilbertson (2002) infected lettuce plants with X. campestris pv. Vittians, which causes bacterial leaf spot; they concluded that the pathogen had the capacity to enter and translocate within the vascular system of lettuce plants without inducing visible disease symptoms. Seeds produced from diseased lettuce plants were externally contaminated at a level of about the 2% incidence of X. campestris pv. vittians, but internally the seeds were not infected. In this case it seems that the pathogen was stopped at the seed surface, which could suggest a kind of communication between bacteria and plant host. Bacterial cell-to-cell or bacteria host communication was hypothesized by Espinoza-Urgel, Salido, and Ramos (2000) when they restored the seed adhesion capacity of Pseudomonas putida KT2440 by mutating the ddcA of this strain, which codes for a putative membrane polypeptide. The expression of ddcA was revealed to be dependent on cell density, on the addition to a conditioned medium and on seeds exudates, suggesting the existence of a quorum-sensing system in this strain.

Strains belonging to the genera Pseudomonas and Rahnella were isolated from surface sterilized seeds of Norway spruce (Cankar et al., 2005). Strains belonging to the same genera were also found in surface-sterilized seeds of yellow lupine (Barac et al., 2004). Both genera represent well-known plant-associated bacteria with growth-promoting properties and biological control potential. It can be hypothesized that endophytic bacteria residing inside the seeds could serve as vectors for the transmission of plant beneficial traits. To validate this hypothesis, we tested if the endophytic bacteria found in the seeds of tobacco could have a beneficial effect on their host plants, especially when grown in “stressing” environments with heavy metals present. More specifically, we examined the effects of the seed endophytes on plant growth and nutrients uptake, and on plant–metal interactions for Nicotiana tabacum grown on Cd-contaminated soils. Tobacco was chosen for its known high metal-accumulating capacity and the fact that it is being tested at several phytoextraction field experiments in Switzerland and Belgium.

MATERIALS AND METHODS

Seed Collection

The seeds of Nicotiana tabacum cv. Badischer Geudertheimer (BaG) used in this study were collected from plants grown on a metal-enriched sandy loamy soil in Rafz, near Zurich.
Airports in Switzerland. The seeds sown in Rafz were obtained from the Landesanstalt für Pflanzenbau, Rheinstetten, Germany. On the Rafz site a phytoextraction-based remediation experiment is running; the soil was contaminated by the application of domestic and industrial sewage sludge in the 1960s. The total metal contents in the soil in 2004, the year of seed collection, were 505, 0.7, and 362 mg kg\(^{-1}\) for Zn, Cd, and Pb, respectively.

**Cultivation of Plants**

Seeds of the BaG were completely sterilized. The seed endophytic bacteria mainly seem to inhabit the endosperm; this allowed a complete sterilization of the seeds without damaging the plant embryo. In the sterilization protocol used, the seeds were submerged for 30 s in 70% ethanol, then were rinsed once in sterile Millipore water for 30 s. Subsequently, the seeds were placed for 35 min in HOCl\(^{-}\) 42% and then were rinsed three times for 10 min in sterile Millipore water and dried using sterile filter paper. The seeds’ sterility was checked by incubating some of them for 3 days at 30°C on 10-times diluted 869 medium (Mergeay et al., 1985), containing per liter distilled water: 1 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 0.1 g D-glucose, and 0.0345 g CaCl\(_2\)\cdot2H\(_2\)O (pH 7). Seeds were considered sterile when no bacterial growth was observed after seed germination.

The seeds were incubated on a Petri dish containing MSM0 medium (Sigma, St. Louis, MO, USA) in the plant-growth chamber for 3 d in the dark followed by 2 d at normal growth-chamber conditions [constant temperature of 25°C (day)—17°C (night), relative humidity of 60–65%, and a 12-h (day) photoperiod provided by Philips TDL 58WT33 fluorescent tubes (photosynthetic active radiation 160 µmol m\(^{-2}\) s\(^{-1}\))]]. Germinated seeds were moved into sterile Magenta (Sigma) containing 100 mL of MSM0 medium (pH 5.6) supplemented with 5.5 g agar per liter. Depending on the test conditions, 10 µM CdCl\(_2\) was added to the medium and/or 56.6 µL of the bacterial inocula was plated on the surface of the solidified medium. The Cd exposure concentration was determined based on two independent preliminary range-finding experiments. At 10 µM CdCl\(_2\), a slight but significant growth inhibition was observed. Cadmium was chosen since it is the metal of major concern present in the Rafz soil, taking into account food chain contamination and risk for human health. It also was remarkable that a high percentage of the bacteria isolated from the seeds were Cd tolerant, while none of them showed Zn tolerance. One plant per Magenta and five replicas (Magenta) were used for each combination inoculum/Cd exposure.

All the plants were kept under complete sterility in the growth chamber for 2 wk; after this period, the covers were removed and replaced with a sterile tissue that allowed gas exchange. The plants were left to grow for 2 more weeks before being harvested. One plant for each combination of inoculum and/or Cd exposure was used to check for the survival of the inoculum in the plant. Three plants were used to analyze plant heavy metals uptake while all plants were used to determine biomass production.

**Seeds’ Surface Sterilization and Extraction of Endophytic Bacteria**

The seeds were surface sterilized for 30 min using a solution of 1% active chloride supplemented with 1 droplet Tween 80 per 100 mL of solution and were rinsed three times with sterile Millipore water for 10 min. To check surface sterility, the seeds were rolled on 10-fold diluted 869 medium and were subsequently removed. To test for the presence and also extraction of seed endophytes, the same seeds were squeezed in a sterile mortar after
adding a sterile 10-mM MgSO₄ solution. Samples dilutions were plated on 10-times diluted 869 medium and were incubated for 7 d at 30°C. Table 1 describes the main characteristics of the cultivable bacteria isolated from the surface-sterilized seeds.

**Preparation of Bacterial Inocula**

Individual strains, combinations of the most representative cultivable strains (Sₐ, Sₚ, Sₚ, Sₖ, and Sₜ), and the endogenous consortium (Sₜ) consisting of both cultivable and noncultivable endophytes were used for inoculation of sterilized *N. tabacum* seeds. The strains were grown at 30°C on a rotary shaker in 10-times diluted 869 medium (Mergeay et al., 1985). Cells were harvested after approximately 12 h at a density of 10⁶ CFU/ml (OD₆₆₀ of 0.5) by centrifugation, washed twice in 10 mM MgSO₄, and resuspended at a density of 10⁷ CFU/mL. Defined consortia were composed by mixing equal volumes of individual cultures.

Cultivable bacteria that are present in the environment account for only 1–5% of the total bacteria (Amann, Ludwig, and Schleifer, 1995). For this reason also, the entire extracted endogenous bacterial consortium, including the uncultivable bacteria, was used for inoculation. This approach was used to get an idea of the general effect of the bacteria that inhabit the seeds on plant growth in the presence and absence of Cd stress. The solution taken directly from the seeds, referred to as Sₜ, was obtained by squeezing 35 surface sterilized tobacco seeds in 2 mL of MgSO₄ and was immediately used to inoculate sterilized tobacco seeds. Dilutions of all the inocula were plated on 10-times diluted 869 medium and were allowed to grow for a 7-d incubation at 30°C in order to determine the CFU mL⁻¹ inoculum.

**Bacterial Heavy Metal Resistance**

The isolated and purified strains were tested for their heavy metal resistance using 284 medium (Schlegel et al., 1961). This medium contained per liter deionized water: 6.06 g Tris-HCl, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH₄Cl, 0.43 Na₂SO₄, 0.2 g MgCl₂ · 6 H₂O, 0.03 g CaCl₂ · 2 H₂O, 40 mg Na₂HPO₄ · 2 H₂O 1%, 10 mL Fe(III)NH₄ citrate solution [containing 48 mg Fe(III)NH₄ citrate per 100 mL], 1 mL microelements solution, and final pH 7. Four different carbon sources—1.3 mL 40% glucose, 2.2 mL 30% gluconate, 2.7 mL 20% fructose, and 3 mL 1M succinate—were added per liter. The microelements solution contained per liter distilled water: 1.3 mL 25% HCl, 144 mg ZnSO₄ · 7H₂O, 100 mg MnCl₂ · 4 H₂O, 62 mg H₂BO₃, 190 mg CoCl₂ · 6H₂O, 17 mg CuCl₂ · 2H₂O, 24 mg NiCl₂ · 6H₂O, and 36 mg NaMoO₄ · 2H₂O. After heat sterilization, the medium was supplemented with different concentrations of CdCl₂ and/or ZnSO₄, both of which are metals that are present in increased concentration at the Rafz site. The minimum inhibiting concentrations for Zn and Cd were determined and defined as the minimal concentration of the heavy metal inhibiting growth of the bacterial strains. Zn concentrations ranging from 0.25 mM to 4 mM and Cd concentrations ranging from 0.15 mM to 1.5 mM were tested.

**Bacterial DNA Extraction**

Genomic DNA extraction was performed as described by Bron and Venema (1972)). DNA extraction failed with four strains, reported as “not determined” (n.d.) in Table 1; this is not unusual, especially when working with strains isolated from natural environments.
Table 1 Characteristics of bacteria used as inocula

<table>
<thead>
<tr>
<th>Strain n°</th>
<th>Composed by</th>
<th>Sequence result</th>
<th>mM Zn</th>
<th>mM Cd</th>
<th>Box PCR-pattern</th>
<th>Inoculum concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>S_a1</td>
<td>Enterobacter sp.</td>
<td>&lt; 0.25</td>
<td>&lt; 0.15</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_a2</td>
<td>Enterobacter sp.</td>
<td>&lt; 0.25</td>
<td>0.15</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_a3</td>
<td>Xanthomonadaceae</td>
<td>&lt; 0.25</td>
<td>&lt; 0.15</td>
<td>B</td>
<td>2.2 × 10^7</td>
<td></td>
</tr>
<tr>
<td>S_a4</td>
<td>nd</td>
<td>&lt; 0.25</td>
<td>&lt; 0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_a5</td>
<td>nd</td>
<td>2</td>
<td>0.6</td>
<td></td>
<td>Q</td>
<td></td>
</tr>
<tr>
<td>S_b1</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.6</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_b2</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.6</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_b3</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.6</td>
<td>N</td>
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<td></td>
</tr>
<tr>
<td>S_b4</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.3</td>
<td>O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_b5</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>4.5</td>
<td>O</td>
<td></td>
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</tr>
<tr>
<td>S_b6</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.6</td>
<td></td>
<td>3.2 × 10^7</td>
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<tr>
<td>S_b7</td>
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<td>0.6</td>
<td>R</td>
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<td>S_b8</td>
<td>Enterobacter sp.</td>
<td>&lt; 0.25</td>
<td>0.6</td>
<td>R</td>
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</tr>
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<td>S_b9</td>
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<td>0.3</td>
<td>O</td>
<td></td>
<td></td>
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<tr>
<td>S_b10</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.45</td>
<td>O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_b11</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.6</td>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_b12</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.6</td>
<td></td>
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<td></td>
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<tr>
<td>S_c1</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.6</td>
<td></td>
<td>N</td>
<td></td>
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<tr>
<td>S_c2</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.6</td>
<td></td>
<td>O</td>
<td>6.7 × 10^7</td>
</tr>
<tr>
<td>S_c3</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.6</td>
<td></td>
<td>O</td>
<td></td>
</tr>
<tr>
<td>S_c4</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.6</td>
<td></td>
<td>O</td>
<td></td>
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<tr>
<td>S_d1</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.3</td>
<td></td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>S_d2</td>
<td>Sanguibacter sp.</td>
<td>&lt; 0.25</td>
<td>&lt; 0.15</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>S_d3</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td></td>
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<tr>
<td>S_d4</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td></td>
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</tr>
<tr>
<td>S_d5</td>
<td>Stenotrophomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.3</td>
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<td></td>
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<tr>
<td>S_d6</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.3</td>
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<td></td>
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<tr>
<td>S_d7</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.6</td>
<td></td>
<td>L</td>
<td></td>
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<tr>
<td>S_d8</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.6</td>
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<td>L</td>
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<tr>
<td>S_d9</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.6</td>
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<tr>
<td>S_d10</td>
<td>Clostridium aminovalericum</td>
<td>&lt; 0.25</td>
<td>0.9</td>
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<td>S_d11</td>
<td>Enterobacter sp.</td>
<td>N.G.</td>
<td>N.G.</td>
<td>I</td>
<td>2 × 10^2 (only the cultivables)</td>
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<tr>
<td>S_d12</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>1.5</td>
<td></td>
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<td></td>
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<tr>
<td>S_d13</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.45</td>
<td>G</td>
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<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>0.75</td>
<td>G</td>
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<td>0.3</td>
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<td>0.9</td>
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<td>0.45</td>
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<td>&lt; 0.15</td>
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<td>Pseudomonas sp.</td>
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<td>0.3</td>
<td>E</td>
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<tr>
<td>S_d20</td>
<td>nd</td>
<td>N.G.</td>
<td>N.G.</td>
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<td>1.05</td>
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<td>&lt; 0.25</td>
<td>&lt; 0.15</td>
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<td>D</td>
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<td>S_d23</td>
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<td>0.45</td>
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<td>2.3 × 10^8</td>
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<td>3 × 10^8</td>
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<td>0.45</td>
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<td>5 × 10^7</td>
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<td>S_d26</td>
<td>Pseudomonas sp.</td>
<td>&lt; 0.25</td>
<td>1.5</td>
<td></td>
<td></td>
<td>7 × 10^8</td>
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</table>

(Continued on next page)
Table 1 Characteristics of bacteria used as inocula (Continued)

<table>
<thead>
<tr>
<th>Strain n</th>
<th>Composed by</th>
<th>Sequence result</th>
<th>mM Zn</th>
<th>mM Cd</th>
<th>Box PCR-pattern</th>
<th>Inoculum concentration</th>
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<tbody>
<tr>
<td>S_e</td>
<td>S_d17</td>
<td>Enterobacter sp.</td>
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<td>6.3 × 10^7</td>
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<td>Pseudomonas sp.</td>
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<td>S_d17</td>
<td>Enterobacter sp.</td>
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<td>2.7 × 10^8</td>
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<td>S_d12</td>
<td>Pseudomonas sp.</td>
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</tr>
<tr>
<td>S_d13</td>
<td>Pseudomonas sp.</td>
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S_d refers to the whole consortium containing both cultivable and uncultivable bacteria. The minimum inhibiting concentrations values for Cd and Zn (mM) are reported. Similar to column reports the BOX-PCR results resumed, similar letters corresponds to similar PCR fingerprinting that corresponds to equal bacterial species. The last column presents the different inocula concentrations as were used in the inoculation experiments. n. d. = not determined.

DNA precipitation was obtained by adding a 1/10 volume of 3M Na Acetate pH 6 and 2.5 × 100% ethanol (−20°C). The DNA was subsequently fished out and solubilized in ddH₂O. DNA quality and quantity were determined on 0.8% agarose gel.

**Box-PCR Genomic DNA Profile**

BOX-PCR was used to discriminate between the different cultivable isolates. The Polymerase Chain Reaction (PCR) contained 5 µL 10× Taq-Buffer, 4 µL 10 mM dNTPs, 2 µL Box1-primer (5′-CTACGGCAAGGCGACGCTGACG-3′), 0.25 µL Taq polymerase (1 unit; Invitrogen), and 5 µL template, in a total final volume of 50 µL. The thermocycling conditions were: 1 min at 95°C, 35 cycles of 1 min at 95°C, 1.5 min at 50°C, 8 min at 65°C, and a final incubation step at 65°C for 8 min. The obtained PCR products were separated by means of a 2% agarose gel electrophoresis, after which their distinct patterns were used to discriminate between the different strains.

**16S rRNA Amplification and Sequencing**

The 16S rRNA gene was amplified using primers P0 (27f sequence: 5′ GAGAGTTTG ATCCTGGGCTCAG) and P6 (1495r sequence: 5′ CTACGGCTACCTTGTTACGA) in a PCR reaction, consisting of: 5 µL buffer (10×), 2 µL MgCl₂ (50 mM), 1 µL of each primer (10 µM each, Invitrogen), 1 µL 10 mM dNTP’s (Invitrogen), 0.4 µL Taq polymerase (Invitrogen), prepared as a master mix, with the addition of sterile ddH₂O until the final volume of 50 µL, prior to DNA addition (1 µL). Cycling conditions were: 1.5 min at 95°C, five cycles of 30 sec at 94°C, 30 sec at 60°C, 2 min at 72°C, five cycles of 30 sec at 94°C, 30 sec at 55°C, 2 min at 72°C, 25 cycles of 30 sec at 94°C, 30 sec at 50°C, 2 min at 72°C, and a final incubation step at 72°C for 10 min (Picard et al., 2000).

The 5 µL of the resulting PCR–DNA product was checked by gel electrophoresis on a 0.8% agarose gel. The remaining PCR products were cleaned by GFX PCR DNA and a gel band purification kit (Amersham Biosciences, Buckinghamshire, UK) before sequence, using the Prism Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) with 100 ng of template DNA. The extended sequences were obtained with universal primers 26F and 1392R. DNA sequences were determined on a 16 Capillary DNA
Sequencer (Applied Biosystems). Taxonomic classifications were determined according to Wang et al. (2007) at the Ribosome Database Project II (http://rdp.cme.msu.edu/index.jsp).

**Plant Sampling**

Plant material was collected, keeping the shoots and the roots of the same plant separated, and was vigorously washed with deionized water. The roots were washed with 10 mM Pb(NO₃)₂ at 4°C for 10 min in order to remove the adhering metals and was rinsed three times with distilled water. The plants’ fresh weight was determined on an analytical balance (Mettler H54). The dry weight was obtained after drying the shoots and roots during 48 h at 60°C.

**Plant Metal Analysis**

Dry plant materials of shoots and roots were separately homogenized and mineralized by wet ashing with a mixture of concentrated HNO₃ and HClO₄ (5:2 v/v). Zn, Cd, and Fe contents were determined by inductively coupled plasma optical emission spectroscopy. The total Cd content of roots and shoots (in micrograms) was calculated by multiplying the plant Cd concentration with the dry weight of these organs.

**Inoculum Recovery**

Plants were harvested after 4 wk of growth; leaf, stem, root, and rhizosphere samples were kept separately. Plants were removed out of the Magenta under sterile conditions and put into a sterile Petri dish after removing the excess growth medium. Five milliliters 10 mM MgSO₄ was added to root material and, after vigorous shaking for 1 min, 100 µL of this solution and its subsequent dilutions were plated to check the inoculum’s survival in strict contact with the root system, considering it as rhizosphere. The biggest leaf was steriley cut from the plant and surface sterilized using 0.1% active chloride supplemented with 1 droplet of Tween 80 per 100 mL solution for 2 min and was rinsed three times with sterile Millipore water. The leaf was dried using sterile filter paper. In order to verify the efficiency of the sterilization, a 100-µL sample of the third rinsing water was plated on 10-times diluted 869 medium. The same procedure was used for stem and root with a 0.5% concentration of active chloride and a sterilization time of 1 min for the stem and 2 min for the roots. After sterilization, the different plant parts were macerated into 10 mM MgSO₄ using a sterile mortar. Samples (100 µL) and their dilutions were plated on 10-times diluted 869 medium and checked after 7 d for growth at 30°C.

**Statistical Analysis**

All treatments were performed with a minimum of three replicas. The significance of the differences was analyzed by one-way and factorial analysis of variance (ANOVA), followed by the Tukey test or the HSD-Tukey test for post-hoc comparisons between unequal samples, using the Statistica 6 software package (StatSoft Version 2003). Using the same statistical program, a Mann–Whitney U test was used in the case of nonnormal distribution. The data referred to the concentration, ratios, and metal contents that were...
RESULTS

All bacteria tested successfully colonized the plants. Results for the biomass production, plant Cd concentration and content, iron–cadmium ration, and Cd translocation factor are presented as percentage values in comparison to those obtained for the noninoculated control plants, which were considered to be 100%.

Effects of Cd Exposure and Endophytic Inoculation on Plant Biomass Production

Growth parameters (roots and shoots dry weight) were determined in order to evaluate the effects of both Cd exposure and endophytic inoculation on plant development. The analyses were made on 4-wk-old plants grown in Magenta pots.

Positive effects on the shoot and/or root biomass production were mainly observed after inoculation by the consortia (Figure 1). Inoculation with the S_f consortium improved the biomass production of shoots and roots of both Cd-exposed and control plants; the S_e consortium had a positive effect on the weight of shoots and roots of Cd-exposed plants and the S_c consortium only had a positive effect on the shoots and roots of control plants. Inoculation with consortium S_d increased the root weight in the absence of Cd (Figure 1), while in the presence of Cd there was no observed effect on the shoot dry weight. On the other hand, inoculation with consortium S_b decreased biomass of the shoots of the Cd-exposed plants.

Inoculation with Sanguibacter sp. S_d2 improved the development of both shoots and roots of Cd-treated plants, resulting in a statistically significant difference with the control plants, while Pseudomonas sp. S_d12 and S_d13 slightly increased shoots fresh weight under Cd stress. Inoculation with the other strains did not result in any significant effect.

Effect of Inoculation on Cd Uptake

In order to evaluate possible effects of endophytic inoculation on Cd uptake and distribution in N. tabacum, metal concentrations and contents were determined in the shoots and roots of inoculated and noninoculated plants exposed and not exposed to Cd.

Knowing that Cd is phytotoxic (Steffens, 1990; Sandalio et al., 2001), we intended to find a relation between biomass production and Cd concentration. Some clear differences between inoculated and noninoculated plants could be observed (Figure 2). Inoculation with both consortia S_d and S_a lead to increased Cd concentrations and also total Cd content in the shoots. Interestingly, after inoculating with the Cd-sensitive strain Sanguibacter sp. S_d2 (Table 1), a slightly lower Cd concentration, but a higher total Cd content was found in the shoots, probably as a result of an increased biomass production (Figure 2). On the contrary, inoculation with Pseudomonas sp. S_d13, which is more tolerant to Cd than S_d2 (Table 1), resulted in a slightly decreased plant Cd concentration (Figure 2).

Since we initially focused on the potential effect of endophytic inoculation on the Cd content in the easily harvestable aerial plant parts, analysis of the roots was only performed during the last experiment. In the final phase of the work, we widened the objective to
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**Figure 1** Biomass production dry weight of shoot (A) and root (B) in plants with different inocula. (Inocula are specified under each histogram.) Histograms with shading refer to the plants grown in presence of 10 µM Cd. Means and standard errors are given of three different biological replicas. The data are expressed as percentage of the two respective controls, considering the controls as 100% (horizontal line splitting the graphs). The statistical significance of the results, marked by a star on the histogram, shows a statistical difference from the noninoculated control plants evaluated on the pure data; statistical differences have been calculated in comparison to the respective control values. The statistical significance of the results was confirmed at the 5% level using a two- and one-way ANOVA models separately exploring inoculums and the presence or absence of Cd with noninoculated plants. The U Mann-Whitney test was performed when ANOVA analyses were not allowed.

all possible effects that the inocula could have on plant growth and elements uptake; thus, root metal content was analyzed as well. In general, after inoculation a decreased root Cd concentration was found. Roots of plants inoculated with *Sanguibacter sp. S_d2* showed a lower Cd concentration, while roots of plants inoculated with *Enterobacter sp. S_d17* had
Figure 2  Cd concentration in shoots (mg kg$^{-1}$ dry weight; A) and in roots ($\mu$g per plant; B) of plants grown in the presence of 10 $\mu$M Cd. The different inocula are specified under each bar. Means and standard errors are shown. The data are expressed as percentage of the respective noninoculated plants (control) considered as 100% (horizontal line splitting the graphs). Indicated by a star on the histogram are the values showing a statistical difference from the noninoculated control plants evaluated on the pure data confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

a lower Cd concentration and total Cd content (Figure 3). Inoculation with Pseudomonas sp. S_d13 and the consortium S_f lowered the plant root Cd concentration (Figure 3).

**Ratios Metal—Cd**

Disturbances in the uptake and distribution of macro- and micronutrients in plants were also shown to be correlated with Cd toxicity (Sandalio et al., 2001). Calculating the ratios between metals, more specifically Fe, with Cd was intended to determine a preferential accumulation of a specific metal in comparison with noninoculated plants. This would suggest an effect of inoculation on metal distribution. It is known that the
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Figure 3 Total Cd content in shoots (µg per plant; A) and roots (µg per plant; B) of plants grown in the presence of 10 µM Cd. The different inocula are specified under each bar. Means and standard errors are shown. The data are expressed as a percentage of the respective noninoculated plants (control) considered as 100% (horizontal line splitting the graphs). Indicated by a star on the histogram are the values showing a statistical difference from the noninoculated control plants evaluated on the pure data confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

Uptake of different nutrients improves the growth of plants on contaminated soils (Belimov *et al.*, 2004; Burd, Dixon, and Glick, 2000; Verkleij and Schat, 1990). Therefore, it is relevant not only to determine the plants’ metal concentrations, but also to check if the ratios of essential elements and Cd change in inoculated plants compared to noninoculated control plants.

*Nicotiana. tabacum* inoculated with *Sanguibacter* sp. S_d2 (Figure 4) showed a lower Fe/Cd ratio in the shoots as compared with noninoculated plants. This means that when the Cd concentration in shoots increased, at least partly, there was a negative effect on the Fe concentration. On the contrary, the Fe/Cd ratio is much higher in the roots of all the inoculations tested. This suggests that the plants, relatively, assimilated more Fe than the...
The iron–cadmium ratio in shoots (A) and roots (B) of plants grown in the presence of 10 µM Cd. The different inocula are specified under each bar. Means and standard errors are shown. Here the data are expressed as a percentage of the respective noninoculated plants (control) considered as 100% (horizontal line splitting the graphs). Indicated by a star on the histogram are the values showing a statistical difference from the noninoculated control plants evaluated on the pure data confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

Figure 4 The iron–cadmium ratio in shoots (A) and roots (B) of plants grown in the presence of 10 µM Cd. The different inocula are specified under each bar. Means and standard errors are shown. Here the data are expressed as a percentage of the respective noninoculated plants (control) considered as 100% (horizontal line splitting the graphs). Indicated by a star on the histogram are the values showing a statistical difference from the noninoculated control plants evaluated on the pure data confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

toxic Cd and that this was more pronounced in the inoculated plants than in the sterile control (Figure 4).

Translocation Factor (TF)

The TF is defined as the ratio between the metal concentrations in the shoot and the metal concentration in the roots. Especially endophytic bacteria, but eventually also rhizosphere bacteria, may have effects on the translocation of metals from roots to shoots. In Cd-exposed *Nicotiana tabacum*, inoculation with *Sanguibacter* sp. *S.d2*, *Enterobacter* sp. *S.d17*, *Pseudomonas* sp. *S.d12*, and the consortium *S.f* increased Cd translocation,
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Figure 5 Translocation Factor of Cd in plants grown in the presence of 10 µM Cd. The different inocula are specified under each bar. Means and standard errors are given of three different biological replicas. The data are expressed as a percentage of the respective noninoculated plants (control) considered as 100% (horizontal line splitting the graphs). Indicated by a star on the histogram are the values showing a statistical difference from the noninoculated control plants evaluated on the pure data confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.

Compartmentalizing more of this metal in the shoots than in the roots (Figure 5). At the same time, Sanguibacter sp. S.d2 decreased the Zn translocation, resulting in an increased amount of this metal in the roots (data not shown).

DISCUSSION AND CONCLUSIONS

In this article, different parameters that should help to better delineate the role played by endophytic bacteria were analyzed, which are naturally present inside tobacco seeds, in the complex interaction with their host plant and trace elements present in the environment.

In this study, inoculation of N. tabacum with both single endophytic strains and consortia did not have a negative effect on plant biomass production. The only exception was observed after inoculation with consortium S.b, which seemed to increase plant sensitivity to Cd, showing a decreased shoots biomass production under Cd stress. In some cases a positive effect of endophytic inoculation on plant growth was observed, even when plants were exposed to Cd (Figure 1). Inoculation with consortia resulted in more pronounced beneficial effects on biomass production (Figure 1) as compared with inoculation with single strains, suggesting synergistic effects of the different members of the consortia. Similar plant-growth–promoting effects by endophytic bacteria were observed with yellow lupine (Barac et al., 2004) and poplar (Taghavi et al., 2005). This suggests that none of the strains is pathogenic and that some of them (e.g., strain S.d2 and consortia S.a, S.c, S.e and S.f.) could be considered as plant growth promoting (PGP). In addition, an increased plant Cd concentration, as compared with noninoculated control plants, was observed several times (Figure 2), suggesting that these bacteria can somehow reduce Cd toxicity through their interaction with plant growth.

It is known that, under imbalanced nutrient conditions, PGP bacteria are key elements in plant establishment. Their use can support eco-friendly crop production that favors a
reduction in the use of agrochemicals (Herrera, Salamanka, and Barea, 1993; Glick, 1995; Requena et al., 1997). The means by which PGP bacteria can improve the nutrient status of their host plant includes: 1) biological nitrogen fixation (diazotrophy); 2) increased nutrient availability into the rhizosphere through the solubilization of unavailable minerals; and 3) increased plant biomass production via the synthesis of phytohormones. Encouraging data have been achieved by Egamberdiyeva and Höflich (2004), who demonstrated that selected PGP bacteria isolated from the soil of different crop root zones (cotton, wheat, tomato, melon, and alfalfa) were able to increase the growth and nutrient uptake of cotton and pea in nutrient-poor Calciisol soil, as compared with the control plants that performed poorly under the same conditions.

There is plenty of evidence for bacterial production of phytohormones. Auxins and cytokinins were found to be produced by strains of *Pseudomonas*, *Enterobacter*, *Staphylococcus*, *Azotobacter*, and *Azospirillum*. These substances, together with gibberellins, may be considered as causal agents for altering plant growth and development (Arshad and Frankenberger, 1991; Bashan and Holguin, 1997; Leifert, Morris, and Waites, 1994). In addition, PGP bacteria can overcome growth inhibition caused by heavy metal stress via the metabolization of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene (Glick et al., 1994; Glick, Penrose, and Li, 1998; Glick, 2004). The strains used in our experiments (Table 1) were not yet investigated for their eventual phytohormone and ACC-deaminase production.

The majority of the cultivable bacteria that were isolated from the seeds (Table 1) belonged to the pseudomonads. The study of their Box-PCR products (Table 1) revealed that in many cases they represented distinct species. Therefore, it is reasonable to expect different results from their inoculations. Some of the inocula studied probably can influence nutrient uptake under nutrient-imbalanced conditions. In the case of S_c, for example, we observed a more than 2-times increased Zn uptake in Cd-treated shoots (data not shown). This increased Zn uptake might explain the decreased Cd toxicity that is evidenced by an increased biomass production (Figure 1). A similar effect was observed after inoculation by strain S_d13 that decreased Zn (data not showed) and Cd concentrations in roots (Figure 2) but at the same time increased Fe concentration in this part (Figure 4). Our data suggest that the root is an important organ for interactions between trace elements. A preferred Fe uptake in this plant part was observed for all the inoculations studied while this was found only in one case for the shoot (Figure 4).

Most of the inoculations lead to an increase in the Cd translocation factor (Figure 5). The plants seem, due to the inoculations, to store more toxic metal into their aerial parts. This suggests that Cd accumulated in the leaves should be less harmful for the plant. However, subcellular localization and speciation of Cd in the leaves were not investigated. In any case, this increased metal-translocation factor is interesting from the point of view of increasing the efficiency of phytoextraction and, therefore, some of these inocula should be tested in a field trial.

In conclusion, it is evident that, even under conditions of Cd stress, seed endophytic bacteria can have a beneficial effect on both biomass production and trace elements (Ca, Fe, Zn) uptake and distribution by their host plant. The results also indicate that it is recommendable to study and use these bacteria as consortia, not only as individual strains. The bacteria composing the consortium, in fact, prove to have a general positive influence on plant development. Of the 10 inocula tested, only S_b had a negative effect on biomass production and this was only in the case of Cd-exposed plants. In general, the inocula either as single strains, defined consortia, or whole consortia seem to lower Cd uptake, especially
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into the root part. In case inoculation resulted in an increased Cd uptake into the shoots, inoculation seemed to reduce Cd toxicity by increasing the uptake of essential nutrients from the growth medium. Our findings for endophytic bacteria isolated from tobacco seeds support the suggestion by Cankar et al. (2005) that the seeds can serve as a vector for beneficial bacteria. Many of the endophytic strains used in this study, such as Sanguibacter sp. S_d2 and Pseudomonas sp. S_d12, as well as the consortia such as S_a, S_c, S_d, S_e, and S_f, seem to be promising candidates for phytoextraction field trials. All of them increased biomass production and Cd content in the plants, especially in the shoot part; good biomass yield and high metal accumulation in harvestable plant parts are essential key factors for efficient phytoextraction (McGrath and Zhao, 2003; Vassilev et al., 2004).

Some important needs for further study on these seed endophytes are as follows:

1. A more detailed study should be performed on the changes that occur in the endophytic seed community in the function of storage time of the seeds to understand if and what changes occur in the composition of the seed endophytic community and how these changes influence plant development.
2. Another open question is the fate of the seed endophytes during development of the plant. For instance, will these seed endophytes become a major fraction of the newly established endophytic community or will they be outcompeted by the microbial population present in the soil once the start to germinate on a soil?
3. Also, the composition of the next generation of seed endophytes should be investigated in function of the plant-growth regime and the composition of the total endophytic community as part of a population-dynamics approach.
4. The improved plant Cd tolerance and plant metal translocation suggest a field experiment using these strains as inocula.

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