Fluorescent pseudomonads occuring in *Macrotermes subhyalinus* mound structures decrease Cd toxicity and improve its accumulation in sorghum plants

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Abstract

Cd-tolerant bacterial strains of fluorescent pseudomonads, mostly belonging to *Pseudomonas monteilii*, were isolated from termite mound soil (*Macrotermes subhyalinus*, a litter-forager and fungus-growing termite), in a Sudanese shrubby savanna, Burkina Faso. Such large mounds appeared as sites of great bacterial diversity and could be considered as hot spots of metal-tolerant fluorescent pseudomonads. Microbial isolates were inoculated to Sorghum plants (*S. bicolor*) in glasshouse experiments with soil amended with CdCl₂ (560 mg Cd kg⁻¹ soil). Microbial functional diversity was assessed at the end of the experiment by measurement of *in situ* patterns of catabolic potentials. All the bacteria isolates significantly improved the shoot and total biomass of sorghum plants compared to the control. Results concerning root biomass were not significant with some strains. Arbuscular mycorrhiza (AM) was greatly reduced by CdCl₂ amendment, and fluorescent pseudomonad inoculation significantly increased AM colonisation in the contaminated soil. The bacterial inoculation significantly improved Cd uptake by sorghum plants. Measurement of catabolic potentials on 16 substrates showed that the microbial communities were different according to the soil amendment. Soils samples inoculated with pseudomonad strains presented a higher use of ketoglutaric and hydroxybutiric acids, as opposed to fumaric acid in soil samples not inoculated. It is suggested that fluorescent pseudomonads could act indirectly in such metabolic processes by involving a lower rate of degradation of citric acid, in line with the effect of small organic acid on phytoextraction of heavy metals from soil. This is a first contribution to bioremediation of metal-contaminated sites with soil-to-plant transfer, using termite built structures. Further data are required on the efficiency of the bacterial strains isolated and on the processes involved.

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Keywords: Heavy metals; Termite; Phyremediation; Fluorescent pseudomonads; Arbuscular mycorrhizal fungi

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1. Introduction

Agricultural practices such as agrochemical usage, long-term application of urban sewage sludge and human industrial activities cause accumulation of metals or metalloids (i.e. arsenic, cadmium, copper, zinc, nickel, mercury). They generally affect aquatic and terrestrial ecosystems and induce potential health risks due to soil-to-plant transfer of metals (Kabata-Pendias, 1992; Khan et al., 2000). Cadmium is one important element for food-chain contamination and is an extremely significant pollutant (Pinto et al., 2004).

Various physico-chemical and biological remediation technologies have been developed during the last decades (Mulligan et al., 1999, 2001a; Wu et al., 2006). In many cases, these processes of heavy metals remediation are usually expensive and labour extensive (Khan, 2005). Hence, alternative and cheaper technologies have to be developed to recover the degraded land, particularly in developing countries. One of these innovative technologies includes plants to clean up metal-contaminated sites by extracting metals from the soil and concentrating them in the aboveground biomass (Salt et al., 1995; Brooks, 1998). This technology emerged as an ecological-friendly alternative to conventional methods (Arduini et al., 2006). However, metal-accumulating plants have to be cultivated for a relatively long time with a number of growth cycles to remove metal-enriched biomass (Felix, 1997; Wenzel et al., 1999). The interactions of heavy metals with microorganisms have therefore been of increasing interest because of their potential to directly remove directly heavy metals from polluted soils and to improve the transfer of metals to aboveground biomass of higher plants (Guo et al., 1996; Burd et al., 2000). The metals lead to the establishment of tolerant microbial populations usually belonging to Bacillus, Arthrobacter and Corynebacterium genera of gram-positive bacteria and Pseudomonas, Alcaligenes,Ralstonia and Burkholderia genera of gram-negatives (Kozdrój and van Elsa, 2001; Ellis et al., 2003). It has also been assessed that arbuscular mycorrhizal fungi (AMF) frequently reduced plant uptake and/or decreased the phytotoxic effects of soil heavy metals (Hildebrandt et al., 1999; Chen et al., 2003a) even if they could enhance uptake of toxic metals in other situations (Weissenhorn et al., 1995).

Innovative techniques based on microbial inoculants need to screen, identify and select bacterial and/or fungal isolates for their potential application in bioremediation of contaminated sites. Large quantities of viable microbial inocula have to be produced after that, which requires an appropriate technical knowledge. In addition, the survival of inoculated bacteria was usually not sustained in subsequent crops and it implied repeated inoculant application. The high cost and technical requirements of this process could limit its use especially in developing countries.

It has recently been demonstrated that termite mound materials, used as natural microbial inoculants, promoted plant growth and increased nutrient supplies from organic and inorganic soil amendments through their effects on soil microorganisms (Duponnois et al., 2005). Among different mound materials tested, fluorescent pseudomonads have only been detected in the termite mounds of Macrotermes subhyalinus (a litter-forager termite) (Duponnois et al., 2005, 2006). Although numerous studies have reported that fluorescent pseudomonads could bend heavy metals efficiently (Minz et al., 1996; Khan, 2005; Piotrowska-Seget et al., 2005), the potentialities of these bacterial species inhabiting the termite mounds of M. subhyalinus are unknown.

The objectives of this study were to determine (i) the proportion of cadmium-tolerant bacteria among the total fluorescent pseudomonad population in the termite mound and (ii) the potentialities of some fluorescent pseudomonads isolates to increase Cd uptake by plants and reduce its toxicity in sorghum plants.

2. Materials and methods

2.1. Screening for Cd-tolerant fluorescent pseudomonads

Soil samples from five termite mounds were collected in a Sudanese shrubby savanna in Burkina Faso near the village of Yaktenga (50 km north of Ouagadougou). They were crushed and passed through a 2 mm-sieve before use. The chemical and microbiological analyses have been reported in a previous study (Duponnois et al., 2005). 10 g fw per sample were placed in Erlenmeyer flasks containing 100 ml of 0.1 M magnesium sulfate (MgSO4, 7H2O) and shaken at 180 rpm for 30 min. Serial 10-fold dilutions of homogenized suspensions were then plated onto King’s B medium in order to detect and count fluorescent pseudomonads under UV light (King et al., 1954). This medium was amended with 2, 4 and 6 mM Cd added as CdCl2. The plates were incubated at 28 °C for 48 h prior to counting. The isolates of fluorescent pseudomonads were randomly selected (10 bacterial strains called KRM1 to KRM10) from the King’s B plates amended with 6 mM Cd, purified, subcultured on the same nutrient agar medium as before and cryopreserved at −80 °C in glycerol 60%-TSB (Tryptic Soy Broth, 3 g L−1. Difco laboratories) culture [1/1, volume in volume (v/v)].
2.2. Bacterial inoculum

The phylogenetic analysis of fluorescent pseudomonads showed that most of them belonged to the *Pseudomonas monteilii* species (Duponnois et al., 2006). The bacterial isolates were grown in 0.3% (m/v) TSB liquid medium for 3 days at 28 °C on a rotary shaker. Then, they were centrifuged (2400 g, 20 min) and suspended in 0.1 M magnesium sulfate. The final concentration of the bacterial suspension was about $10^8$ CFU mL$^{-1}$ after enumeration on a plate count agar medium (King’s B medium). This suspension was used as inoculum.

2.3. Glasshouse experiment

Seeds of sorghum (*Sorghum bicolor* L.) were surface-sterilized with 1% NaOCl for 15 min and rinsed with demineralised water. They were pre-germinated for 2 days in Petri dishes on humid filter paper at 25 °C in the dark. The germinating seeds were used when rootlets were 1–2 cm long.

The soil used was collected from a non-cultivated area near Ouagadougou (Burkina Faso). Its chemical and physical characteristics were as follows: pH (H$_2$O) 5.6; clay, 4.6%; fine silt, 0.0%; coarse silt, 0.8%; fine sand, 25.5%; coarse sand, 69.1%; total carbon, 0.204%; total nitrogen, 0.04%; soluble phosphorus 0.043 mg kg$^{-1}$; total phosphorus, 0.116 mg kg$^{-1}$. One part of the soil was autoclaved (140 °C, 40 min) and the other was not. Cd was added as chloride. The soil was treated with a single dose of 560 mg Cd kg$^{-1}$ soil. The salt (CdCl$_2$) was dissolved in 1 L distilled water and mixed thoroughly with the soil (both autoclaved and non-autoclaved) according to Kozdrój (1995).

Sorghum plants were individually grown in 0.1 L pots filled with the autoclaved or non-autoclaved soil and amended with Cd. Immediately after planting, the young seedlings were inoculated with 5 ml bacterial suspension. Control treatments without bacteria received 5 ml of 0.1 M magnesium sulfate. Control treatments without CdCl$_2$ amendment and without bacterial inoculation.

1 month after microbial inoculations, the plants were harvested and their root systems gently washed under running tap water. The oven dry weight (1 week at 65 °C) of the shoot was measured. In order to quantify the colonization of arbuscular mycorrhizal fungi, the roots were cleared and stained according to the method of

![Graph](image)

Fig. 1. Number of Cd tolerant fluorescent pseudomonads on King’s B agar medium contaminated with increasing CdCl$_2$ amendments. Error bars represent standard errors. Data of columns indexed by the same letter are not significantly different according to the Newman–Keuls test ($p<0.005$).

Table 1

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Shoot biomass (mg dry weight)</th>
<th>Root biomass (mg dry weight)</th>
<th>Total biomass (mg dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disinfected soil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−CdCl$_2$</td>
<td>208.3 (6.31) (1)b (2)</td>
<td>191.4 (15.5) b</td>
<td>399.7 (19.1) b</td>
</tr>
<tr>
<td>+CdCl$_2$</td>
<td>24.9 (1.68) a</td>
<td>34.1 (3.81) a</td>
<td>58.9 (4.21) a</td>
</tr>
<tr>
<td>Non-disinfected soil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−CdCl$_2$</td>
<td>200.6 (12.94) b</td>
<td>97.9 (10.58) b</td>
<td>298.5 (20.14) b</td>
</tr>
<tr>
<td>+CdCl$_2$</td>
<td>24.8 (2.24) a</td>
<td>9.9 (0.83) a</td>
<td>34.6 (2.76) a</td>
</tr>
</tbody>
</table>

(1) Standard error of the mean. (2) Data in the same column and for each factor followed by the same letter are not significantly different according to the Newman–Keuls test ($p<0.05$).
Phillips and Hayman (1970). They were cut into 0.5 cm short pieces and placed on a slide for microscopic observations at 250× magnification (Brundrett et al., 1985). The entire root system was observed for each plant. The extent of mycorrhizal colonization was expressed as (the number of mycorrhizal root pieces)/(number of non-mycorrhizal root pieces) × 100. Then, for each plant, root pieces were put together and their dry weight was measured (1 week at 65 °C).

The soil from each pot was carefully mixed and kept at 4 °C for further analysis. Microbial functional diversity in soil treatments with non-autoclaved soil was assessed by measuring the patterns of in situ catabolic potential (ISCP) of microbial communities (Degens and Harris, 1997). These measurements were made from 3 soil samples randomly chosen in each microbial treatment. Fifteen carboxylic acids (glutamic acid; tartaric acid; gluconic acid; citric acid; ketobutyric acid, uric acid; oxalic acid;...
succinic acid; gallic acid; mannose; ascorbic acid; quinic acid; ketoglutaric acid; hydroxybutyric acid; fumaric acid) and one carbohydrate (D-glucose) were screened for differences in SIR responsiveness between soil treatments. Their concentrations providing optimum SIR responses were 100 mM for carboxylic acids and 75 mM for glucose (Degens and Harris, 1997). 1 g equivalent dry weight soil was mixed with each substrate suspended in 2 ml sterile distilled water (West and Sparling, 1986) in 10 ml bottles. CO₂ production from basal respiratory activity in the soil samples was also determined by adding 2 ml sterile distilled water to 1 g equivalent dry weight of soil. Bottles were closed immediately after the addition of the substrate solutions to soil samples and kept at 28 °C for 4 h. CO₂ fluxes from the soils were measured using an infrared gas analyzer (IRGA) (Polytron IR CO₂, Dräger™) in combination with a thermal flow meter (Heinemeyer et al., 1989). Results were expressed as μg CO₂ g⁻¹ soil h⁻¹.

For the analysis of leaf Cd contents, 50 mg of dried leaves (1 week at 60 °C) were ground in a porcelain capsule, ashed (2 h at 450 °C) in a muffle furnace and cooled down. At ambient temperature, the ash was suspended in 2 ml HCl 6 N and then in 5 ml distilled water. The suspension subsequently filtered through a quantitative filter paper. Then they were analysed by atomic absorption spectrophotometer flame using a Varian Spectra AA200FS analytical system.

2.4. Statistical analysis

The data were treated with one- and two-way analysis of variance. Means were compared using the Newman and Keuls test (p<0.05). The percentages of mycorrhizal colonization were transformed by arcsin(sqrt) before statistical analysis.

Between-Group Analysis (BGA, Dolédec and Chessel, 1987, 1989; Culhane et al., 2003) was used to analyse the SIR responses in soil samples amended with cadmium and inoculated with various pseudomonads strains. Three groups of samples were considered: control (Cd−), Cd amendment (Cd+), and Cd amendment with fluorescent pseudomonad inoculation (Bact). BGA is a multivariate analysis technique derived from principal components analysis (PCA). The aim of PCA is to summarize a data table by searching orthogonal axes on which the projection of the sampling points (rows of the table) have the highest possible variance.

From a theoretical point of view, BGA is the particular case of PCA with respect to instrumental variables (PCAIV, Rao, 1964; Lebreton et al., 1991) where the instrumental variables table is reduced to just one qualitative variable. This variable defines groups of rows in the data table, and BGA consists of the PCA of the table of the means by groups. This table has a number of rows equal to the number of groups, and the same number of columns as the original table. The aim of this analysis is to separate the groups. This is also the aim of discriminant analysis (also called canonical variates analysis), but while discriminant analysis is limited to tables that have a high number of samples compared to the number of variables, BGA can be used even when the number of rows is less than the number of variables. BGA can thus be considered as a robust alternative to discriminant analysis when the number of samples is low.

A Monte Carlo test (permutation test) can be used to check the significance of the differences between groups. This method consists in performing many times a random
permutation of the rows of the table (but not of the qualitative variable defining the groups), followed by the re-computation of the between-class inertia. By comparing the between-class inertia obtained in the normal analysis with the between-class inertia obtained after randomisation, we get an estimation of the probability of meeting a situation similar to the observed situation, without differences between groups (i.e., a significance test of the differences between groups).

Computations and graphical displays were made with the free ADE-4 software (Thioulouse et al., 1997), available on the Internet at http://pbil.univ-lyon1.fr/ADE-4/.

3. Results

In the range from 2 mM to 6 mM CdCl₂, the percentages of Cd-tolerant fluorescent pseudomonads varied from 85.1% to 4.2% (Fig. 1). After 1-month culture, the addition of CdCl₂ has significantly decreased the shoot and root growth of sorghum plants in both the disinfected and in the non-disinfected soil (Table 1).

For all microbial inoculations combined, there were no significant differences between the total biomass of sorghum plants in both the disinfected and in the non-disinfected soil (Table 2). However, root biomass was significantly higher in the disinfected soil whereas shoot biomass was higher in the non-disinfected soil (Table 2).

For both disinfected and non-disinfected soil treatments combined, all the bacterial isolates have significantly improved the shoot and total biomass of sorghum plants compared to the control (Table 2). However, with four of them (KRM1, KRM2, KRM3 and KRM5) no significant effects have been recorded on root biomass (Table 2).

No mycorrhizal structures have been detected on the roots of sorghum plants growing in the disinfected soil. In the non-disinfected soil, mycorrhizal colonization by indigenous AM fungi was significantly reduced by CdCl₂ amendment and decreased from 60.2% (non-Cd amended soil) to 20.3% (Cd amended soil without bacterial inoculation) (Fig. 2). Fluorescent pseudomonad inoculation significantly increased AM colonization indexes in the contaminated soil as they ranged from 31.6% (KRM6) to 45.1% (KRM2) (Fig. 2).

In the control treatment, the metal concentration in the aboveground biomass was 3.11 mg per plant (Fig. 3). The bacterial inoculation significantly improved Cd uptake by plants excepted for the KRM8 fluorescent pseudomonad isolate (Fig. 3). Metal contents per plant ranged from 5.7 (KRM1) to 10.7 mg per plant (KRM7) (Fig. 3). In addition, measurements of Cd concentrations in sorghum shoot were significantly higher in the bacterial treatments (from 97 mg kg⁻¹ to 189 mg kg⁻¹) than in the control (90 mg kg⁻¹).

BGA of the SIR responses with respect to the three soil types appeared on Fig. 4. The map of soil samples (Fig. 4B) showed that the three types (Cd−, Cd+, Bact) were clearly separated, which showed that the microbial communities were different according to the soil amendment. The Monte Carlo test was significant (p=0.025). The map of the 16 substrates (Fig. 4A) showed that, on the first axis, the use of
glutamic acid was higher in the control samples (left part of the figure). Conversely, CO₂ production was globally lower in soil samples amended with Cd (right part of the figure). The second axis opposed fumaric acid (up) to ketoglutaric and hydroxybutyric acids (down). Soil samples inoculated with pseudomonad strains presented a higher use of these two acids, while fumaric acid use was higher in soil samples not inoculated with pseudomonad strains.

Cd concentrations in the aboveground biomass of sorghum plants were negatively correlated with SIR responses with glutamic acid \( (r=0.52, p=0.02) \), citric acid \( (r=0.38, p=0.02) \), fumaric acid \( (r=0.32, p=0.05) \) and gluconic acid \( (r=0.39, p=0.02) \).

### 4. Discussion

It has been previously assessed that many soil bacteria were intrinsically resistant to high concentrations of heavy metals (Gadd, 1992; Landmeyer et al., 1993; Angle et al., 1993). For instance, a single 5 mg Cd g⁻¹ introduction into the soil reduced the number of heterotrophic bacteria (from \( 10^7 \) cfu g⁻¹ to \( 10^6 \) cfu g⁻¹ of dry soil). Enumeration of metal tolerant bacteria from contaminated soils have been realized in numerous studies (Kandeler et al., 2000; Ellis et al., 2003; Dell’Amico et al., 2005). Piotrowska-Seget et al. (2005) have showed that about 50% of the culturable bacteria isolated from sandy-loam soil contaminated with heavy metals were Cd-tolerant. This high percentage should be explained by the impact of long-term metal exposure involving the establishment of tolerant bacterial populations. Moreover, Piotrowska-Seget et al. (2005) have counted the Cd metal tolerant bacteria using a nutrient agar medium amended with 0.5 mM Cd. In the present study, the termite mound structure has never been contaminated with Cd and almost all the \( P. \) monteilii isolates were tolerant to a higher Cd concentration (2 mM). Hence \( M. \) subhyalinus mound structure could be considered as a natural hot spot of metal-tolerant bacteria, especially for the fluorescent pseudomonad group. This study confirmed that termite mounds could be sites of great bacterial and fungal diversity (Meiklejohn, 1965; Mohindra and Mukerji, 1982; Holt, 1998; Rouland-Lefèvre et al., 2002). Higher microbial diversity recorded in termite structures, was generally attributed to higher levels of organic matter and a better supply of nitrogen (Mohindra and Mukerji, 1982), as well as to higher moisture levels and higher substrate availability (Abbadie and Lepage, 1989).

Fluorescent pseudomonad inoculation significantly increased the total biomass of sorghum plants and it seemed it was very effective in protecting plants from growth inhibition caused by Cd contaminants. Hence, \( P. \) monteilii isolates could be considered as Plant-Growth Promoting Rhizobacteria (PGPR). PGPR stimulate plant development directly by providing bio-available phosphorus for plant uptake, sequestering trace elements (Iron) for plants by siderophores, producing plant hormones (Glick et al., 1999). It is known that gram-negative copiotrophic organisms as pseudomonads are strongly stimulated by root exudates (Kozdrój and van Elsas, 2000). Pseudomonads were found to be efficient in bioaccumulation of heavy metals in polluted conditions (Hussein et al., 2001, 2005). Such organisms are producing anionic biosurfactants (i.e. rhamnolipids in \( P. \) aeruginosa) removing metals from contaminated soils (Mulligan et al., 2001b).

It has also been also reported that soil microorganisms could directly contribute to plant establishment by immobilizing heavy metals in rhizosphere and by reducing subsequent plant uptake (Channugathas and Bollag, 1987). In general, with bacterial inoculation alterations of metal concentration in plant tissues are not very significant (Wu et al., 2006). In the present study, bacterial inoculation increased the Cd content of the shoots. Although soil Cd content has not been measured, as the loss of Cd through water lixiviation has not been controlled, this result suggested that bacterial inoculants increased the Cd bio-availability.

In addition, these bacterial isolates significantly increased AM formation and acted as Mycorrhiza Helper Bacteria (MHB). It has been previously reported that a MHB, isolate of \( P. \) monteilii (isolate HR 13), stimulated AM colonization of Acacia holosericea seedlings by Glomus intraradices (Duponnois and Plenchette, 2003). In that study, it has also been reported that bacterial inoculant alone had no effect on the growth of Australian Acacia species. Hence, in the present study, positive bacterial effects on the plant growth could result from two main mechanisms: (i) the higher development of AM fungi enhanced supply of essential nutrients from the soil to the host plant and reduced phytotoxic effects (Hetrick et al., 1994; Hildebrandt et al., 1999; Joner and Leyval, 2001; Chen et al., 2003a) and (ii) bacterial inoculant enhanced Cd bio-availability and plant Cd uptake. These data support previous studies in which the use of indigenous AM fungi and MHB, inoculated together, is recommended in order to restore metal contaminated soils (reviews in Leyval et al., 1997; Khan, 2002, 2004).

The patterns of in situ catabolic potential (ISCP) of microbial communities showed that Cd amendment affected their functional abilities as it has been found in numerous studies (Kandeler et al., 2000; Ellis et al., 2003).

The mechanisms controlling the mobility of metals in soil are generally subjected to organic compounds that play...
an essential role by chelating metals and, consequently, influence their solubility in the soil horizons (Brynhildsen and Rosswall, 1989; Krishnamurti et al., 1997). Among the organic compounds in soil solution, the low-molecular-weight organic acids belonging to the non-volatile aliphatic category (e.g. citric and oxalic acids) are of special interest because of their ubiquitous occurrence and high metal-complexing capacity (Stevenson, 1967; Wildung et al., 1979). Such organic acids enhance the mobilisation of metals through weathering and chelation (Grayston et al., 1996; Chen et al., 2003b). Furthermore, low molecular weight organic molecules may carry metal ions and increase cell membrane permeability (Pizzeghello et al., 2000; Pinto et al., 2004). Chelating agents, such as EDTA and citric acid have been used as a viable technology for mobilizing lead (Wu et al., 1999) and zinc (Luo et al., 1992). The role of citric acid on the availability, accumulation and detoxification of Cd was studied by Chen et al. (2003b). They concluded that this organic acid converted the cadmium into more easily transported forms and stimulated its transportation from root to shoot. In the present study, the SIR responses with citric acid were negatively correlated with the Cd amounts in the shoots. Hence, microorganisms, able to catabolize this organic acid were less abundant. It suggested that fluorescent pseudomonad inoculants acted indirectly in these biological processes by involving a lower rate of degradation of citric acid, as it has been described by Evangelou et al. (2006) on the effect of small organic acids on phytoextraction of Cu and Pb from soil with tobacco plants.

In conclusion, this study confirmed that microbial communities that inhabit Macrotermiteinae-built structures are of great genetic and functional diversity that could be used for different topics such as phytoremediation processes. The processes involve interactions between the rhizosphere microflora and plant roots, a key question that need to be better understood (Kozdroy and van Elsas, 2000). Further use in technologies employing plants to treat metal-contaminated sites requires more information such as its efficiency on other heavy metals.

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References


