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ORIGINAL ARTICLE

# GFP-tagged multimetal-tolerant bacteria and their detection in the rhizosphere of white mustard

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**Abstract** The introduction of rhizobacteria that tolerate heavy metals is a promising approach to support plants involved in phytoextraction and phytostabilisation. In this study, soil of a metal-mine wasteland was analyzed for the presence of metal-tolerant bacterial isolates, and the tolerance patterns of the isolated strains for a number of heavy metals and antibiotics were compared. Several of the multimetal-tolerant strains were tagged with a broad host range reporter plasmid (i.e. pPROBE-NT) bearing a green fluorescent protein marker gene (*gfp*). Overall, the metal-tolerant isolates were predominately Gram-negative bacteria. Most of the strains showed a tolerance to five metals (Zn, Cu, Ni, Pb and Cd), but with differing tolerance patterns. From among the successfully tagged isolates, we used the transconjugant *Pseudomonas putida* G25 (pPROBE-NT) to inoculate white mustard seedlings. Despite a significant decrease in transconjugant abundance in the rhizosphere, the *gfp*-tagged cells survived on the root surfaces at a level previously reported for root colonisers.

**Keywords** Bacterial inoculation · GFP-tagged strains · Metal tolerance · Mine soil · Rhizosphere colonisation

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## Introduction

The introduction of rhizosphere-competent bacteria that are able to tolerate increased concentrations of heavy metals is a promising approach for the improvement of phytoextraction and phytostabilisation carried out by some metal-tolerant plants (Kuiper et al. 2004; Lebeau et al. 2008). Plant inoculation with the released bacteria has to be accompanied with the monitoring of their survival in the rhizosphere. Therefore, inoculants should be tagged with a marker that allows the introduced cells to be identified and monitored among the populations of indigenous soil microorganisms. One of the more useful methods to do this relies on the *gfp* gene, which encodes the green fluorescent protein (GFP) from the jellyfish *Aequora victoria* (Errampalli et al. 1999). The unique feature of this marker is its exclusive suitability for monitoring released bacteria in soils because the *gfp* is absent in soil microorganisms, and the expression of green fluorescence does not require any substrate or cofactor (Unge et al. 1998; Cassidy et al. 2000; Kozdrój et al. 2004). In addition, the *gfp* marker enables the researcher to study the introduced strains in situ with a minimum of sample preparation, thus avoiding possible disturbance of the natural cell colonisation pattern.

GFP-tagged bacteria have been used to inoculate the soil and the seeds/seedlings of plants exposed to high levels of

heavy metals (Liao et al. 2006; Braud et al. 2009; Ma et al. 2009). The major goal of such studies is to identify the conditions affecting both the survival of the released mutants and their activity in terms of the stabilisation of metal concentrations in a habitat. Beneficial bacterial inoculants are selected to support the growth of plants; these generally show tolerance to heavy metals and have phytoextraction or phytostabilisation activity (Wu et al. 2006a; Braud et al. 2009). This results in the establishment of relevant plant–microorganism associations that are highly compatible in removing metal from soil (Lebeau et al. 2008). Metal-mine wastelands, which standardly contain high concentrations of heavy metals, such as zinc (Zn), copper (Cu), lead (Pb), nickel (Ni), and cadmium (Cd), can be rich sources of metal-resistant bacteria and metal-resistant plants (Barrutia et al. 2011) for bioaugmentation-assisted phytoextraction and phytostabilisation. For example, Ma et al. (2009) reported that the Cu-resistant strain of *Achromobacter xylosoxidans* significantly improved Cu uptake by metal-accumulating Indian mustard (*Brassica juncea*) and promoted plant growth. However, before any bacterial strain can be used for soil bioaugmentation-assisted phytoremediation, its survival, persistence and habitat colonisation, as well as its ability to interact with a plant host must be assessed.

White mustard (*Sinapis alba*) belongs to a diverse group of mustards that show an increased tolerance to heavy metals. These plants have been suggested for application in the phytoextraction or phytostabilisation of the metals in contaminated soils (Wu et al. 2006b; Lebeau et al. 2008). Reporter broad host range plasmids of the pPROBE series containing GFP gene have been employed to construct bacterial metal-biosensors (Liao et al. 2006) or inoculants used for bioaugmentation of a metal-contaminated soil (Braud et al. 2009). We report here the first trials to introduce these plasmids into multimetal-tolerant bacterial isolates from the soil of a metal-mine wasteland. The aim of the research was to determine which of the *gfp*-marked multimetal-tolerant bacterial strains were able to survive in the rhizosphere and on the roots of white mustard. These trials represent the first step to assess an application potential of the plant–bacteria association in a metal-contaminated environment.

## Materials and methods

### Source of metal-tolerant bacteria

A composite soil sample, prepared from eight different subsamples taken from an area of 25 $m^2$, was collected from the top layer (0–10 cm) of a metal-mine wasteland in Piekary Slaskie. The site is located in Upper Silesia, an industrialised region of southern Poland. The soil (pH 7.04, organic matter 2.6%) contained high total concentrations of Zn, Pb, Cu, Cd and Ni (i.e. 30, 11, 2.8, 0.2 and 0.09 mg $g^{-1}$ dry soil, respectively). The concentrations were determined by atomic absorption spectrometry (UNICAM 939/959) after wet-mineralisation in a mixture of $HNO_3$ (4 ml) and $H_2O_2$ (1 ml), using a microwave oven (Piotrowska-Seget et al. 2005).

To isolate metal-tolerant bacteria, triplicate soil samples (10 g) were placed in Erlenmeyer flasks, each containing 90 ml of 0.1% sterile sodium pyrophosphate (pH 7.0), and shaken at 120 rpm for 30 min. Serial tenfold dilutions of these soil suspensions were plated onto one-tenth strength trypticase soy broth agar [0.1× TSBA: tryptic soy broth (TSB; Difco, Detroit, MI) 3 g, agar 15 g $l^{-1}$, pH 7.0] and King's B agar (Bacto peptone 20 g, $K_2HPO_4$ 1.5 g, $MgSO_4 \cdot 7H_2O$ 1.5 g, glycerol 10 ml, agar 15 g, demineralised water 1 l, pH 7.2) amended with 3 mM Zn (as chloride). The plates were incubated at 24°C for 5 days (Rasmussen and Sørensen 2001) followed by subculturing of representative colonies differing in morphology and colour onto fresh agar plates. Bacterial isolates were selected from the $10^{-3}$ and $10^{-4}$ dilutions. A total of 25 isolates were selected and used for identification, using Gram staining and analysis of cellular fatty acid methyl esters (FAMEs).

### Identification of bacterial isolates and their tolerance patterns

We extracted FAMEs (MIDI Inc, Newark, DE) from each isolate, using the standard and recommended procedure that included saponification, derivatisation, extraction and final base washing (Microbial ID Inc. 1999). The organic phase, containing cellular FAMEs, was separated using a HP 6890 gas chromatograph (GC; Hewlett Packard, Palo Alto, CA) on a Ultra 2-HP capillary column with hydrogen as the carrier gas and analysed by Sherlock 4.0 MIDI software, using the aerobe TSBA40 method and TSBA40 library (MIDI Inc). The MIDI system generated a similarity index (SI) for each strain as a mark of the confidence with which the isolate was identified. Strains with an SI level >0.3 were considered to be identified (Germida and Siciliano 2001; Piotrowska-Seget et al. 2005).

We determined the metal tolerance pattern of each metal-tolerant strain by the minimum inhibitory concentration (MIC) approach. To this end, 0.1× TSBA plates, amended with increasing (from 1 to 10 mM) levels of Zn, Cu, Cd, Ni or Pb, were inoculated with bacterial strains and incubated at 24°C for 3 days. The MIC was defined as the lowest metal concentration preventing the growth of the strains.

To establish an antibiotic tolerance of the isolated bacteria, each strain was plated onto 0.1× TSBA supple-

mented with ampicillin (Ap; 100 μg $ml^{-1}$), tetracycline (Tc; 20 μg $ml^{-1}$) or kanamycin (Km; 20 μg $ml^{-1}$) followed by incubation at 24°C for 3 days. The isolates growing on respective plates were considered to be antibiotic tolerant.

### Tagging with GFP of bacterial isolates

Two different strains of *Escherichia coli* were used in a triparental conjugation to introduce *gfp* into selected metal-tolerant bacterial isolates. The promoter-probe strain of *E. coli* (pPROBE-NT), harbouring a *PnptII::gfp* fusion (Miller et al. 2000), was used as the source of a mobilisable plasmid, containing a red-shifted *gfp* gene associated with a Km resistance gene. In addition, used *E. coli* (pRK2013) resistant to Km (Figurski and Helinski 1979) as the donor of a self-transmissible plasmid pRK2013 that mobilised pPROBE-NT to the recipient isolates.

The procedure of triparental mating consisted of three steps. First, 0.5-ml aliquots of *E. coli* (pPROBE-NT) (optical density at 560 nm: 0.6) were mixed with 0.5 ml of *E. coli* (pRK2013) (optical density at 560 nm: 0.8) and concentrated by centrifugation for 5 min at 2,700 *g*. The bacterial pellet was then suspended in 0.5 ml of sterile saline followed by the centrifugation for 1 min at 2,700*g*. Second, a 1-ml aliquot of a recipient isolate (optical density at 560 nm: 0.9) was added to the pellet, mixed and centrifuged for 1 min at 2,700 *g*. The bacterial pellet was then suspended in 0.1 ml of sterile saline followed by plating onto LB agar (Difco, pH 7.2). The inoculated plates were incubated at 37°C for 24 h. Third, the transconjugant cultures were thoroughly scraped from the plates and suspended in 2 ml of sterile saline. The suspensions were serially diluted up to $10^{-3}$ followed by plating (0.1 ml) onto 0.1× TSBA supplemented with Ap (100 μg $ml^{-1}$), Km (20 μg $ml^{-1}$) and 3 mM Zn (as chloride). As transconjugant controls, the cultures of donor or particular recipients (optical density at 560 nm: 0.8) were also spread on a similar medium amended with the antibiotics and the metal. All plates were incubated at 27°C for 24–48 h until distinct colonies were seen. Finally, we restreaked selected colonies on the selection plates to ensure purity and the presence of the markers. Putative transconjugant cells were checked for GFP expression under an Olympus FluoView FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). The microscope was equipped with a multiline argon laser (excitation 488 nm, emission range 500–550 nm) and a water immersion (WI) planapochromatic 60×/1.20 objective.

### Survival of GFP-transconjugants in the rhizosphere

For the survival studies, we chose GFP-tagged *Pseudomonas putida* G25 (pPROBE-NT) isolates that showed the best growth under the selection conditions as well strong and persistent expression of the *gfp*. To this end, we incubated the transconjugant culture overnight with shaking at 28°C until late log phase ($10^9$ CFU $ml^{-1}$), after which the cells were harvested by centrifugation and washed three times in sterile saline before being suspended in 10 ml of the saline.

The soil of the metal-mine wasteland was air-dried to about 15% (w/w) moisture content, sieved (mesh diameter 2 mm), placed in plastic containers (300 g) and wetted with distilled water to about 35% (w/w) moisture content. This moisture corresponded to about 50% (w/w) of the water holding capacity of the soil. Prior to the trials, we seeded triplicate soil portions with white mustard (*Sinapis arvensis)*. These were left for 3 weeks to grow in a plant growth cabinet under a light/dark regime (26°C, 16 h/21°C, 8 h) at a relative air humidity of 75%. The tolerance of indigenous culturable bacteria to Ap, Tc and Km was checked by plating serial dilutions of triplicated soil samples (3 g, shaken at 130 rpm for 30 min) onto 0.1× TSBA amended with Ap (100 μg $ml^{-1}$), Tc (20 μg $ml^{-1}$) or Km (20 μg $ml^{-1}$) followed by incubation at 24°C for 6 days.

To inoculate mustard seedlings, we added a suspension of transconjugant *P. putida* G25 (log 7.82 CFU $ml^{-1}$) to the soil surface, establishing a moisture content of about 35%. All pots were placed in the plant growth cabinet under a light/dark regime (26°C, 16 h/21°C, 8 h) at a relative air humidity of 75%.

After 0, 7, 14, 28 and 54 days, white mustard seedlings were carefully removed from the soil, and the roots with adhering rhizosphere soil were placed in sterile 0.1% sodium pyrophosphate (pH 7.0) for shaking (130 rev $min^{-1}$, 30 min) and preparing serial tenfold dilutions. Replicate aliquots from the rhizosphere dilutions were spread-plated onto 0.1× TSBA amended with either Ap (100 μg $ml^{-1}$) or Km (20 μg $ml^{-1}$) for the counting of *P. putida* G25 (pPROBE-NT). The medium without the antibiotics was used to enumerate total indigenous heterotrophic bacteria. The medium was also amended with cycloheximide (Ch; 100 μg $ml^{-1}$) to inhibit the growth of fungi. In addition, to avoid a possible masking effect of indigenous fluorescent *Pseudomonas* spp., we supplemented the selective medium with 0.45 mM $FeSO_4 \cdot 7H_2O$, which represses siderophore biosynthesis (Timms-Wilson and Bailey 2001). Plates inoculated with the transconjugant *P. putida* G25 were analysed after incubation at 24°C for 2–4 days, whereas those for the total heterotrophic bacteria were incubated for 6 days. We enumerated fluorescent colonies containing GFP using a hand-held UV light in a dark room.

Selected white mustard roots with adhering soil were collected for microscopic observations. To this end, the samples of roots and rhizosphere soil were separately transferred to glass vials containing 8 ml of 0.1% sodium

pyrophosphate (pH 7.0). The roots were gently washed for 30 s, while the rhizosphere soil samples (1 g) were vigorously shaken for 5 min. After the large soil particles had settled down (15 min, 18°C), a drop of the upper level of the soil suspension was applied to a microscope slide. The slide was then prepared for observation under a confocal laser microscope (Unge et al. 1998). The root samples were washed three times in saline, and the excised fragments (maximum length: 10 mm) were placed on microscope slides and prepared for observation under the confocal microscope.

### Statistics

The count data of culturable bacteria were subjected to the analysis of variance (ANOVA) followed with post hoc comparison of means using Fisher's least significant difference test (LSD, $n$=3, $p$<0.05). To compare the patterns of tolerance to the metals and antibiotics among the isolated bacterial strains, we used a joining method of the cluster analysis (CA) module (Statistica ver 6.0; StatSoft, Tulsa, OK) and Ward's clustering algorithm. Thus, a dendrogram showing clustering trends among all isolates was created.

## Results and discussion

### Isolation of metal-tolerant strains from metalliferous soil

Soil of a metal-mine wasteland is a habitat rich in heavy metals that exert a strong selecting pressure that enables the growth of different metal-tolerant bacteria. Low concentrations of nutrients and the restricted availability of water and oxygen are additional constraints on the growth of microorganisms in this habitat. It has been reported that Gram-positive bacterial species predominate among the bacteria surviving in soils polluted with high concentrations of heavy metals (Roane and Kellog 1996; Ellis et al. 2003; Åkerblom et al. 2007; Sułowicz et al. 2011). By contrast, other studies have indicated that it is Gram-negative bacteria which predominate in sites rich in heavy metals (Kunito et al. 1997; Brim et al. 1999; Piotrowska-Seget et al. 2005). In total, we isolated 25 Cu-tolerant bacterial strains from the soil collected at the metal-mine wasteland; of these 16 isolates were Gram-negative and nine strains were Gram-positive organisms. With the exception of two isolates, IGB 2 and IGB 8, these metal-tolerant strains were identified to the species level based on their MIDI-FAME profiles, all with SI > 0.600 (Table 1). The highest number (i.e. 10) of strains were identified as *Pseudomonas putida*. This species and other fluorescent pseudomonads have often been reported as organisms of great adaptability to harsh conditions in soil contaminated with heavy metals (Roane 1999; Duponnois et al. 2006; Wu et al. 2006a).

Most bacterial strains revealed tolerance to five metals; however, the tolerance patterns differed among the isolates. Three strains of *Pantoea agglomerans* expressed high tolerance to all the metals. In turn, *Citrobacter diversus* and *Klebsiella pneumoniae* were tolerant to 10 or 9 mM Zn, and the latter strain was also resistant to 3 mM Cd. In addition, the isolate IGB 8 tolerated up to 10 mM of Cu and Ni (Table 1). Overall, most strains tolerated higher concentrations of Zn, Cu and Ni than of Pb and Cd. Similar results were obtained by Piotrowska-Seget et al. (2005) for metal-tolerant bacteria isolated from polluted arable soils and barren spoil of a former silver mine. Multimetal tolerance is a characteristic feature of different heterotrophic bacteria isolated from highly polluted soils (Trojanovska et al. 1997; Malik et al. 2002; Sułowicz et al. 2011). Ryan et al. (2005) found that 82% of isolates from metal-polluted soil showed resistance to five out of eight tested metals. Piotrowska-Seget et al. (2005) also found that plasmid-containing bacteria were tolerant of several metals. Multimetal-tolerant bacterial strains have been found to be able to survive in metalliferous soils planted with some plants showing an increased tolerance to various metals (Epelde et al. 2010; Sułowicz et al. 2011).

In terms of antibiotic tolerance, only five of the identified species (i.e. *Bacillus cereus* GB1, *Arthrobacter oxydans*, *Citrobacter diversus*, *Brevibacterium acetylicum* and *Pseudomonas putida* G17) showed a tolerance to Tc, Kn and/or Ap. A similar number of strains, however, all belonging to one species, namely *P. putida* (i.e. G15, G16, G18, G24 and G25), tolerated only Ap. By contrast, four strains (i.e. *Bacillus sphaericus* GB3, *B. cereus* GB7, *B. amyloliquefaciens* and the isolate IGB 8) were sensitive to all three antibiotics (Table 1). Antibiotic tolerance often accompanies an increased resistance to heavy metals among different bacteria isolated from sites exposed to high pollution and/or containing material rich in the metals (Berg et al. 2005; Stepanauskas et al. 2005; Baker-Austin et al. 2006). This situation results from the co-transfer of antibiotic resistance genes and those of metal resistance on the same plasmid under selective conditions (Foster 1983; Baker-Austin et al. 2006). However, the ecological role of this association for bacterial strains occupying a severe habitat is not fully understood. Presumably, the extra feature of antibiotic resistance increases their survival success during the competition for available niches when in the presence of compounds acting as antimicrobials and signal molecules. As a result, respective strains of bacteria may differ in their tolerance profiles, which may in turn affect their survival (Alonso et al. 2001; Hibbing et al. 2010). Our cluster analysis of the metal and antibiotic tolerance patterns of all the bacterial strains showed that they grouped

**Table 1** Bacterial strains isolated from a metalliferous soil and their patterns of tolerance to selected heavy metals and antibiotics

| Strain | MIC [a] (mM) | | | | | Antibiotics[b] | | |
|---|---|---|---|---|---|---|---|---|
| | Zn | Cu | Ni | Pb | Cd | Ap | Tc | Km |
| *Bacillus cereus* GB1 | 7 | 5 | 6 | 2 | 1 | r | r | r |
| Isolate GB2 | 7 | 6 | 5 | 2 | 1 | r | s | r |
| *Bacillus sphaericus* GB3 | 4 | 6 | 3 | 2 | 0 | s | s | s |
| *Arthrobacter oxydans* | 6 | 6 | 4 | 2 | 1 | r | r | r |
| *Citrobacter diversus* | 10 | 7 | 7 | 1 | 2 | r | r | r |
| *Klebsiella pneumoniae* | 9 | 8 | 7 | 2 | 3 | r | r | s |
| *Bacillus cereus* GB7 | 8 | 6 | 4 | 3 | 0 | s | s | s |
| Isolate GB8 | 8 | 10 | 10 | 3 | 0 | s | s | s |
| *Brevibacterium acetylicum* | 5 | 5 | 6 | 3 | 2 | r | r | r |
| *Bacillus sphaericus* GB10 | 5 | 7 | 6 | 2 | 1 | s | s | r |
| *Bacillus amyloliquefaciens* | 6 | 8 | 7 | 2 | 1 | s | s | s |
| *Pantoea agglomerans* GP12 | 9 | 10 | 6 | 2 | 4 | r | s | r |
| *Pantoea agglomerans* GP13 | 9 | 10 | 6 | 2 | 3 | r | s | r |
| *Pantoea agglomerans* GP14 | 9 | 10 | 6 | 2 | 4 | r | s | s |
| *Pseudomonas putida* G15 | 8 | 4 | 2 | 2 | 2 | r | s | s |
| *P. putida* G16 | 7 | 4 | 4 | 2 | 2 | r | s | s |
| *P. putida* G17 | 8 | 4 | 4 | 2 | 2 | r | r | r |
| *P. putida* G18 | 0 | 2 | 2 | 1 | 2 | r | s | s |
| *P. putida* G19 | 8 | 4 | 4 | 2 | 2 | r | s | s |
| *P. putida* G20 | 8 | 4 | 4 | 2 | 2 | r | s | r |
| *P. putida* G21 | 4 | 3 | 4 | 1 | 2 | r | s | r |
| *P. putida* G22 | 7 | 3 | 4 | 3 | 2 | r | s | r |
| *Comamonas acidovorans* | 7 | 3 | 4 | 3 | 1 | r | s | r |
| *P. putida* G24 | 4 | 2 | 2 | 0 | 1 | r | s | s |
| *P. putida* G25 | 7 | 3 | 2 | 2 | 1 | r | s | s |

[a] Minimum inhibitory concentrations were determined on 0.1× tryptic soy broth (TSA) amended with the metals

[b] The strains are tolerant (r) or sensitive (s) to: ampicillin (Ap; 100 μg ml$^{-1}$), tetracycline (Tc; 20 μg ml$^{-1}$), kanamycin (Km; 20 μg ml$^{-1}$)

into two major clusters (Fig. 1). The first composite cluster included only strains of *Pseudomonas putida*, with the one exception being *Comamonas acidovorans*. However, the second major cluster was composed of two subclusters, with one comprising all of the Gram-positive strains, and the second consisting of Gram-negative strains of *Pantoea agglomerans* (clustering together) and *Klebsiella pneumoniae* grouping with *Citrobacter diversus* separately (Fig. 1). This clustering pattern shows that the separation of the *P. putida* group may have been associated with the possession of a common mechanism(s) of metal tolerance that is chromosomally encoded (Cánovas et al. 2003). In contrast, the Gram-positive strains with their thicker cell envelopes react to biocides differently. Their clustering might be, at least in part, due to the presence of a common plasmid carrying genes for multimetal and drug resistance (Kamala-Kanan and Kui Jae 2008). Bacterial species such as *P. agglomerans*, *K. pneumoniae* and *C. diversus* belong to the same family of *Enterobacteriaceae*. Therefore, they formed the separate cluster towards the other Gram-negative group of *P. putida* in this study. In addition, their likeness supports the fact the multidrug resistance (*mar*) operon is widespread

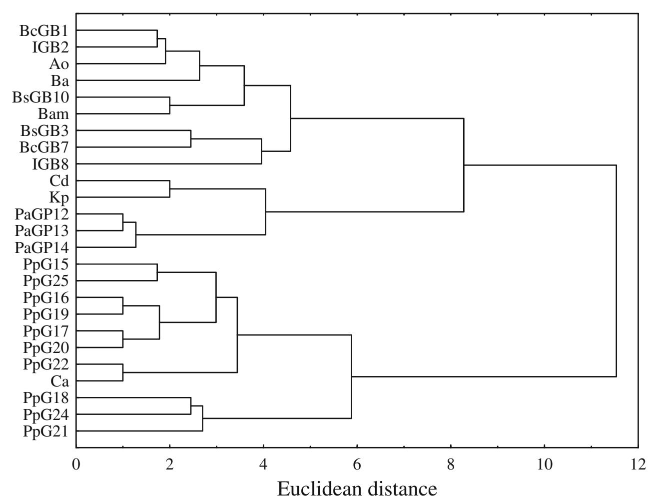


**Fig. 1** Dendrogram representing similarities of metal and antibiotic tolerance patterns of different bacterial strains isolated from soil of a metal-mine wasteland. *Ao Arthrobacter oxydans*, *Ba Brevibacterium acetylicum*, *Bam Bacillus amyloliquefaciens*, *Bc Bacillus cereus*, *Bs Bacillus sphaericus*, *Ca Comamonas acidovorans*, *Cd Citrobacter diversus*, *IGB2*, *IGB8* isolates GB2 and GB8, *Kp Klebsiella pneumoniae*, *Pa Pantoea agglomerans*, *Pp Pseudomonas putida*

among enteric bacteria (Cohen et al. 1993). However, closer clustering of these bacteria with the Gram-positive group may be explained by their similar ability for protection against the biocides, due to the role of the cell envelopes (i.e. glycocalyx and thick cell wall, respectively). Regarding the cluster of *K. pneumoniae* and *C. diversus*, their likeness may have resulted from the presence of the same phosphatase-mediated metal accumulation process involved in the detoxification of the bacteria (Macaskie et al. 1994).

### *gfp*-tagged strains and its survival in the mustard rhizosphere

The underlying rationale for isolating metal-tolerant bacteria from metalliferous soils is their potential application for bioaugmentation-assisted phytoremediation of these habitats. Various markers, often with different detection frequencies, have been used to track the fates of these metal-tolerant bacterial isolates following their reintroduction into the soil (Zaidi et al. 2006; Ma et al. 2009). In our study, we successfully introduced the plasmid pPROBE-NT by triparental conjugation into four strains: *Pseudomonas putida* G16, *P. putida* G20, *P. putida* G25 and *Comamonas acidovorans*. The growth of these strains on selective agar medium containing Ap (100 μg $ml^{-1}$), Km (20 μg $ml^{-1}$) and 3 mM Zn was used to select for the pPROBE-NT transconjugants as neither donor strains nor recipients were able to grow on the medium amended with these markers. We also checked the putative transconjugants for GFP production under the confocal microscope. All transconjugants gave positive results, with *P. putida* G25 (pPROBE-NT) showing the strongest green colour, indicating intensive synthesis of the protein (Fig. 2). Therefore, we used transconjugant strain G25 for further survival experiments. An approach that involves the tagging of bacterial strains with *gfp* genes by plasmid transfer or recombination into the chromosome has been recommended by various authors because of the efficient detectability of the tag and the low energetic burden placed on the cells. (Kendall and Badminton 1998; Errampalli et al. 1999; Kozdrój et al. 2004).

The introduction of metal-tolerant bacterial strains into soil seeded with plants that tolerate increased concentrations of heavy metals has been reported as a promising approach that facilitates the survival and development of these plants in contaminated habitats (Abou-Shanab et al. 2003; Sheng and Xia 2006; Ma et al. 2009). However, the success of this approach is dependent on the potential of the inoculant to colonise plant roots efficiently, which in turn is related to its own survival in the rhizosphere. To estimate the level of adaptation between the released inoculant cells and white mustard, we inoculated plant seedlings growing in a sandy soil. The numbers of *gfp*-tagged *Pseudomonas putida* G25 colonising the roots of white mustard decreased

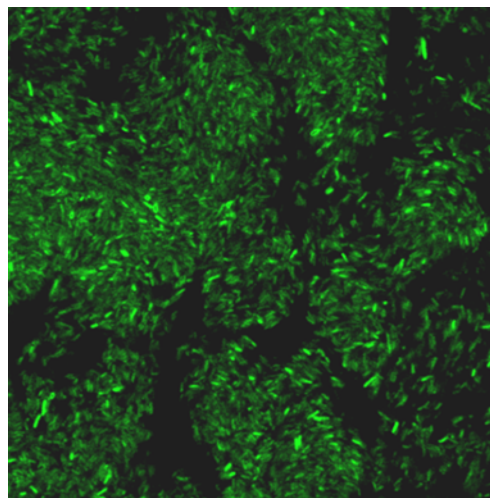

**Fig. 2** Photograph of microscopic image of fluorescent green fluorescent protein (*gfp*)-tagged transconjugant *Pseudomonas putida* G25. The photograph was obtained using a confocal laser scanning microscope

from the initial log 7.48±0.28 to log 4.95±0.25 and log 3.62±0.18 CFU $g^{-1}$ dry soil on days 14 and 54 post-inoculation, respectively. The average counts of the total indigenous heterotrophic bacteria were about log 7.95±0.24 CFU $g^{-1}$ dry soil in the rhizosphere. However, the natural resistance to Ap, Tc and Km among the indigenous bacteria was below the detection limit of log 1.47±0.15 CFU $g^{-1}$dry soil. The microscopic observation of root and rhizosphere preparations confirmed the successful survival of the transconjugant in the rhizosphere and the colonisation of the roots of white mustard seedlings on day 7 (Fig. 3). Although the presence of the *gfp*-tagged transconjugants on the roots was still visible on day 54, only a few bacterial cells were noticeable in the rhizosphere specimen (Fig. 4). A decrease in counts of introduced bacteria over a few days is often observed due to competition for nutrients and space with other rhizosphere microorganisms. They are grazed on by protozoa and exposed to abiotic stress; some cells die or lose culturability following release (van Veen et al. 1997). As a result, the inoculant population ultimately reaches a level reflecting its ability to adapt to conditions prevailing in the rhizosphere of the appropriate plant species (de Weger et al. 1995; Kozdrój et al. 2004). Errampalli et al. (1998) indicated that *gfp*-marked *Pseudomonas* sp., introduced into a creosote-contaminated soil, declined over a 26-day period, although the low numbers recovered up to 13 months after inoculation. It can also not be excluded that the decreased numbers of *P. putida* G25 (pPROBE-NT) may have resulted from the loss of the plasmid over time. However, this vector has been reported to be a stable one in a broad range of bacterial hosts (Miller et al. 2000). Belimov et al. (2004) reported a slight decrease in the numbers of inoculant

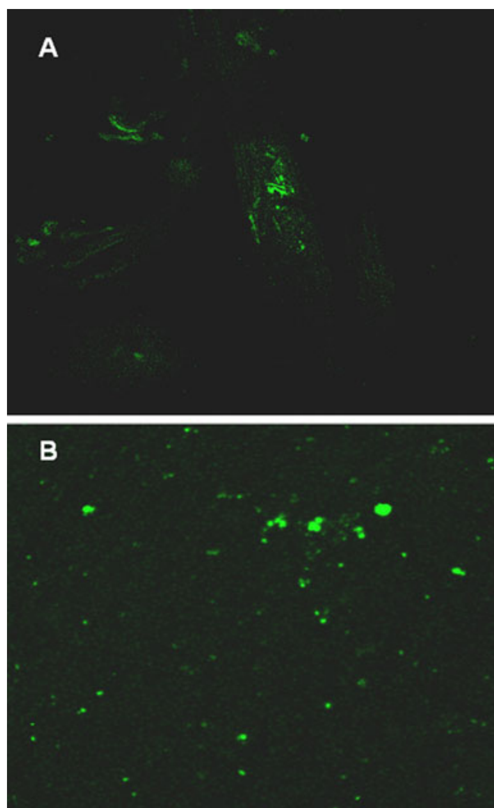


**Fig. 3** Photographs of microscopic images of fluorescent *gfp*-tagged transconjugant *Pseudomonas putida* G25 colonising the root surface of a 7-day-old seedling of white mustard (**a**) and those surviving in the rhizosphere (**b**). The photographs were obtained with a confocal laser scanning microscope

rhizobacteria between days 10 and 25 during their colonisation of barley roots. By contrast, an introduced population of *Bacillus* sp. that was resistant to Cd was still detectable at the same density in the rhizosphere of rape 2 weeks after inoculation (Sheng and Xia 2006). In addition, the survival of inoculants associated with a plant host depends on changes in the physiological state of the plant (Lebeau et al. 2008). Wu et al. (2006b) reported that young mustard seedlings are more favourable to an introduced metal-tolerant strain than flowering plants, possibly due to differences in the composition of the root exudates. We obtained similar results for the survival of *gfp*-tagged *P. putida* G25. Immobilisation of bacterial inoculants into carriers, such as alginate, clay, peat or methyl cellulose, which protects them against biotic and abiotic environmental stress, can increase both their survival in soil as well as their colonisation of soil (van Veen et al. 1997; Kozdrój et al. 2004; Braud et al. 2009). However, to facilitate colonisation of the entire rhizosphere and roots of growing seedlings by the released bacterial strains, the application of free-cell suspensions, instead of immobilised cells, appears to be useful (Ciccillo et al. 2002; Mazolla et al. 1995).

In conclusion, the soil of the metal-mine wasteland is a habitat favouring the selection of multimetal-tolerant heterotrophic bacteria, mostly represented by Gram-negative species, which can be differentiated according to their metal and antibiotic tolerance patterns. Although these bacteria are characterised by their high metal tolerance, only a few strains can be recipients of the *gfp*-bearing reporter plasmid and subsequently express the green fluorescent protein. Indeed, one recipient strain, *Pseudomonas putida* G25, yielded a transconjugant that distinctly expressed GFP and was able to colonise the rhizosphere and roots of white mustard seedlings. Despite a significant decrease in the counts of the transconjugant in the rhizosphere, the *gfp*-tagged cells persist at the level reported for root colonisers (Scher et al. 1994). This result is a promising indicator of plant–transconjugant interdependence that can favour both partners. Further studies are needed to determine whether *P. putida* G25 (pPROBE-NT) can promote the growth of white mustard in soil containing high concentrations of heavy metals, bearing in mind that the ultimate goal is the potential application of both organisms in bioaugmentation-assisted phytoremediation of polluted habitats.

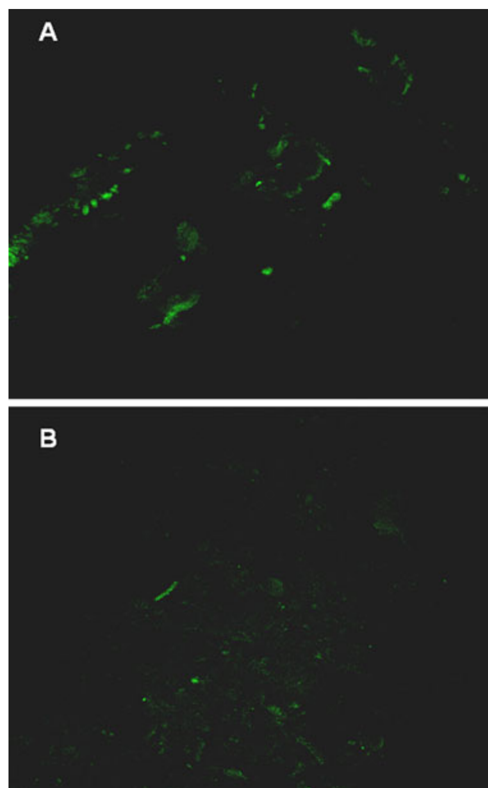


**Fig. 4** Photographs of microscopic images of fluorescent *gfp*-tagged transconjugant *P. putida* G25 colonising the root surface of a 54-day-old seedling of white mustard (**a**) and those surviving in the rhizosphere (**b**). The photographs were obtained with a confocal laser scanning microscope

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