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Gene transfer in bacteria from soils contaminated with heavy metals

K. Lawlor, A.M. Chaudri, S.P. McGrath and P.R. Hirsch
Soil Science Department, IACR-Rothamsted, Harpenden, UK

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K. LAWLOR, A.M. CHAUDRI, S.P. McGRATH AND P.R. HIRSCH. 1999. Transfer of metal resistance plasmids into two pseudomonad recipients, *Pseudomonas aureofaciens* and *Ps. putida*, from soil bacteria donor populations, was investigated in agricultural soil contaminated predominantly with Zn and Cu. The putative donor and recipient numbers on selective agar were not affected by the concentration of metals in the soils, nor were the number of transconjugants. However, there were differences in transfer frequencies of Hg and Cu resistance from the different soil samples. This is the first time that transfer of Cu resistance has been observed from native bacteria present in agricultural soils.

INTRODUCTION

Bacterial gene transfer has been shown to occur in diverse environments including bulk soil (Top *et al*. 1990; Klingmüller 1991), the rhizosphere (Knudsen *et al*. 1988; Lilley *et al*. 1996), the phylloplane (Normander *et al*. 1998), water and the epilithon (Trevors *et al*. 1987; Hill *et al*. 1992). The transfer of plasmids conferring resistance to heavy metals has also been demonstrated in metal contaminated sites such as those near smelters (Top *et al*. 1994), but little work has been done in agricultural soils contaminated with metals (Trevors *et al*. 1987). Many experiments have used metal resistance genes as markers, especially Hg resistance, to select for transconjugants because they are relatively stable and easy to work with (Mergeay *et al*. 1990). However, using genes for resistance to metals as an indicator of bioavailable metals in the environment has not been tested. The diversity of plasmids within a population of *Rhizobium leguminosarum* biovar *trifolii* has been shown to increase in plots amended with sewage sludge with increasing metal concentrations, up to 200 mg Zn kg^{-1} soil and 1·2 mg Cd kg^{-1} soil, in two long-term field experiments at Braunschweig, Germany (Turner *et al*. 1995). Above these concentrations, both the rhizobial populations and the number of plasmid groups decreased, resulting in a sharp fall in the diversity of plasmid phenotypes (Turner *et al*. 1995). These results suggest that plasmid carriage and consequently, gene transfer may be a more sensitive predictor of the effects of metals on bacterial populations than microbial population size.

In this study, gene transfer frequencies were investigated in Zn and Cu contaminated soils from a long-term field experiment to which metal contaminated sewage sludges had been added. The use of gene transfer as a possible sensitive indicator of metal pollution of soil was also investigated. The site investigated had metal-contaminated sewage sludges added in 1982 and 1986. The metals were found to have had a detrimental effect on plant productivity in 1996. The possibility that Zn and Cu resistance gene transfer is increased by high metal concentrations was tested by comparison with transfer of Hg-resistance, for which there was no obvious selection pressure in these soils.

MATERIALS AND METHODS

Soils used

The soil was from a long-term field experiment at ADAS Gleadthorpe in Nottinghamshire, UK to which sewage sludges had been added. This site was first established in 1982 on a sandy loam soil (6% clay, 2% organic matter) of the Newport association (Typic Quartzisament). De-watered and compressed sewage sludge (sludge cake) from a single source was enriched with metal salts of either Zn or Cu. Various amounts of metal-enriched sludge cake were applied to produce a range of soil metal concentrations (Table 1). Control (non-metal enriched) sludges (CS) were used where necessary to make up quantities, so that all sludge plots

Correspondence to: Dr P.R. Hirsch, Soil Science Department, IACR-Rothamsted, Harpenden, Hertfordshire, AL5 2JQ, UK (e-mail: Penny.Hirsch@bbsrc.ac.uk).

Table 1 Mean soil and soil pore water chemical analysis for the different treatments

		Soil pore water		Total soil metal concentration mg kg^{-1}				Soil pore water solution mg l^{-1}		Free soil pore mg l^{-1}	
Treatment	Soil pH	pH	DOC* mg l^{-1}	Zn	Cu	Ni	Cd	Sol Zn	Sol Cu	Free Zn^{2+}	Free Cd^{2+}
No sludge	6·7	6·48	21·37	44	8	7	0·18	0·069	0·01	0·06	0
Control sludge	6·6	6·06	24·89	70	12	7	0·28	0·23	0·02	0·08	0·0001
Low Zn	6	5·19	35·52	308	21	8	0·87	17·78	0·05	7·72	0·0092
High Zn	5·8	5·51	26·53	444	20	9	0·87	17·09	0·04	8·55	0·0073
Low Cu	6·1	6·19	26·24	60	142	8	0·20	0·23	0·20	0·11	0·0009
High Cu	5·8	5·72	32·15	77	364	9	0·27	0·80	0·58	0·31	0·0014

*DOC, Dissolved organic carbon.

received 100 t ha^{-1} dry solids in 1982. In 1986, further additions of sludge cakes, naturally rich in Zn and Cu, were made to selected plots to achieve the required metal concentrations. In August 1997, soil samples from four treatments with single metal sludges, together with an uncontaminated sludge cake control (CS) and a no sludge control (NS) treatment, were used for gene transfer studies.

Soil chemical analysis

Total metals in soil were acid extracted using the method of McGrath and Cunliffe (1985), and determined by inductively coupled plasma atomic emission spectrometry (ICP-AES; Accuris) and Cd by graphite furnace atomic absorption spectrometry (GF-AAS). Water from the soil was extracted using the procedure of Knight *et al.* (1998) and the soluble and free metal concentrations were determined using the procedure of Holm *et al.* (1995).

Soil bacterial analysis

All reagents were made in reverse osmosis deionized water and sterilized. A 1 g sample of soil was suspended in 10 ml Ringers solution (Oxoid) containing 1 g of 2 mm glass beads and vortexed for 10 min. This soil suspension was plated on tryptone soy broth agar (TSBA, Oxoid), and pseudomonad selective agar containing the antibiotics centrimide, fucidin and cephaloridine (CFC) at final concentrations of 10, 10 and 50 $\mu g\,ml^{-1}$, respectively (PSA-CFC, Oxoid), to estimate the number of donor micro-organisms. The agar plates were then incubated at 28 °C for 48 h. The recipient strains used for mating were *Pseudomonas aureofaciens* 381^R (Lilley *et al.* 1994) and *Ps. putida* UWC1 (McClure *et al.* 1989), both of which are rifampicin-resistant. The recipients were grown overnight in the presence of rifampicin, washed three times in sterile Ringers solution and then resuspended in 5 ml Ringers solution. A 1 ml aliquot of resuspended cells was then added to 5 g soil suspended in 7 ml Ringers solution which had previously been vortexed with glass beads for 10 min. The soil and recipient suspension was then thoroughly mixed and 1 ml plated onto nutrient agar (Oxoid) for the transfer of plasmids to occur. For each treatment, two replicate soil samples were used (four for the NS control), and the recipient and transconjugant numbers were measured in triplicate from each of these replicates. Strain UWC1, carrying a transfer positive Hg-resistant 294 kbp plasmid pQBR11, was prepared as the recipient and added to soil at the same time as a positive control for transfer into the recipient 381^R. The mating plates were incubated at 25 °C for 18 h before resuspending some of the bacterial growth into 10 ml sterile Ringers solution, mixing vigorously and plating as follows. Mercury-resistant transconjugants were selected on standard plate count agar (Oxoid) containing 100 mg l^{-1} rifampicin, and 20·1 mg l^{-1} Hg as $HgCl_2$. Copper-resistant transconjugants were selected on yeast extract-glycerol medium (CYEG, 1 g l^{-1} casitone, 0·36 g l^{-1} yeast extract, 2 ml l^{-1} glycerol and 5 g l^{-1} agar, amended with 100 mg l^{-1} rifampicin and with 80 mg l^{-1} Cu added as $CuSO_4$). Zinc transconjugants were selected on CYEG agar containing 100 mg l^{-1} rifampicin and 654 mg l^{-1} Zn as $Zn(NO_3)_2$. Transconjugant numbers for Hg resistance were counted at 48 h, and those for Cu and Zn, 96 h after plating onto the selective agar. All bacterial counts and transconjugant numbers are expressed as cells per gram of oven-dried soil. The transfer frequency was calculated as the number of transconjugants that grew on media containing the respective metal and rifampicin, as a proportion of the numbers of the recipient which grew only on rifampicin. Recipients were counted at the start of the mating and also at the same time as the transconjugants. The recipient strains were also plated onto the medium containing the metal to confirm that the strain itself was not resistant to the metal, and to exclude the possibility that the metal was a mutagen resulting

in a high frequency of spontaneously resistant mutants. Transconjugants were confirmed by streaking them three times on selective media. To confirm the presence of plasmids in the transconjugants, agarose gel electrophoresis was used following a modification of the method of Eckhardt (1978).

RESULTS AND DISCUSSION

The concentrations of both Cu and Zn were elevated in soils receiving metal-contaminated sewage sludges compared with untreated soils (NS) or those treated with uncontaminated sludge (CS) (Table 1). The concentrations of other metals, such as Cd and Ni were low even in the Zn and Cu treatments. The total Zn and Cu in the soil water, and the unbound Zn^{2+} and Cd^{2+}, indicate the bioavailability of these metals in the soils. Mercury is very difficult to measure in soils and its concentration was not determined. However, Hg concentrations are generally extremely low in agricultural soils. The dissolved organic carbon (DOC) in soil water was higher in the CS soils compared with the NS soils, but was of the same order of magnitude in all treatments, reflecting the long-term nature of the experiment. The bulk soil and soil water pH values were within the range normally found in agricultural soils.

The numbers of aerobic heterotrophic bacteria were between 0·96 and $2·0 \times 10^6$ cells g^{-1} soil, and the pseudomonad numbers were between 0·27 and $2·3 \times 10^4$ cells g^{-1} soil in all treatments; neither were significantly different ($P < 0·5$) in the metal-contaminated soils compared with the NS and CS soils. The number of recipients also did not differ significantly ($P < 0·5$) between the treatments after the 28 h incubation period and was between 1 and 5×10^7 g^{-1} soil for 381^R and 0·07 and 5×10^8 g^{-1} soil for UWC1. The mutation frequency of recipient to metal resistance was less than 10^{-8}. Table 2 shows the frequency of transconjugants arising from of the soils. The frequency of UWC1 Hg-resistant transconjugants was small in all treatments, but confirmed that resistance gene transfer occurred. The transfer of Hg-resistance genes is known to occur in the absence of measurable concentrations of Hg in soils (Mergeay *et al*. 1990; Lilley *et al*. 1996). There are relatively large differences in the transfer frequencies observed for each soil replicate, although the triplicate mating from each replicate showed little variation. This possibly reflects the inherent variability in agricultural soils and makes comparison of frequencies between different soils difficult. No Hg-resistant UWC1 transconjugants were observed from either control soil, and Cu- and Hg-resistant transconjugants were not found in any metal-contaminated soils. With the recipient 381^R, the frequency of Cu- and Hg-resistant transconjugants was generally higher in all treatments compared with UWC1 (Table 2). The most marked effects were the relatively higher frequencies of Cu-resistant

Table 2 Transfer frequency of resistance genes from bacteria in soils with different concentrations of Zn and Cu

Recipient strain	UWC1		381^R	
Resistance transferred	Hg	Cu	Hg	Cu
NS, no sludge control	$1·2 \pm 0·1 \times 10^{-5}$	$2·7 \pm 0·3 \times 10^{-4}$	$2·7 \pm 0·3 \times 10^{-4}$	
	ND	$9·2 \pm 3·3 \times 10^{-6}$	ND	ND
	ND	$1·9 \pm 1·1 \times 10^{-5}$	ND	ND
	ND	$1·2 \pm 0·1 \times 10^{-5}$	$4·4 \pm 2·9 \times 10^{-6}$	$4·4 \pm 2·9 \times 10^{-6}$
CS, control sludge	ND	$0·9 \pm 8·1 \times 10^{-6}$	$2·7 \times 10^{-7}$*	$0·8 \pm 1.1 \times 10^{-5}$
	ND	$0·2 \pm 5·4 \times 10^{-6}$	$2·7 \times 10^{-7}$*	ND
Low Zn, 308 mg kg^{-1}	$2·6 \pm 3·4 \times 10^{-10}$	$0·2 \pm 7·0 \times 10^{-6}$	$0·2 \pm 1·7 \times 10^{-8}$	$2·6 \pm 3·4 \times 10^{-6}$
	ND	$0·1 \pm 3·7 \times 10^{-7}$	$1·9 \times 10^{-7}$*	$2·6 \pm 3·4 \times 10^{-6}$
High Zn, 444 mg kg^{-1}	$1·3 \pm 1·7 \times 10^{-10}$	$0·4 \pm 5·8 \times 10^{-6}$	$1·0 \times 10^{-7}$*	$6·5 \pm 6·6 \times 10^{-6}$
	ND	$0·4 \pm 4·1 \times 10^{-5}$	$1·0 \times 10^{-7}$*	ND
Low Cu, 142 mg kg^{-1}	$1·1 \pm 1·0 \times 10^{-9}$	$0·4 \times 10^{-4}$*	$1·8 \times 10^{-7}$*	$2·6 \pm 3·5 \times 10^{-6}$
	ND	$0·4 \times 10^{-4}$*	$1·8 \times 10^{-7}$*	$7·9 \pm 5·6 \times 10^{-6}$
High Cu, 364 mg kg^{-1}	ND	$7·9 \times 10^{-4}$*	$1·0 \times 10^{-7}$*	$4·6 \pm 2·7 \times 10^{-4}$
	ND	$5·5 \pm 1·4 \times 10^{-7}$	$1·0 \times 10^{-7}$*	$9·9 \pm 4·7 \times 10^{-4}$

ND, not detected.

*Standard error $< 0·0001$.

The mean and standard error from three replicates are shown for each soil sample tested.

UWC1 and 381^R transconjugants from the Cu soils. Transfer of Zn resistance from the donor population derived from soil to the two pseudomonad recipients was not detected on agar for any of the treatments. It is not clear whether this reflects the absence of Zn resistance genes on conjugative plasmids in the soil population, or whether mating conditions were unsuitable for transfer and selection.

To confirm that the presence of Hg resistance in UWC1 was due to acquisition of plasmids from the donor population, 30 putative transconjugants were selected at random and analysed for plasmid content by agarose gel electrophoresis. Plasmids were seen in all UWC1 transconjugants from the low Cu, low Zn and high Zn soil, similar to or larger than the 294 kbp plasmid pQBR11 used to ensure conditions were suitable for transfer (gel not shown). We did not attempt to visualize plasmids in the Cu-resistant transconjugants, but our study shows that the donor soil bacterial population in soils moderately polluted with metals could transfer Cu resistance genes to the recipient strains. Further examination of Cu-resistant gene diversity would be very interesting.

In this study, there was no obvious correlation between Hg resistance gene transfer and metal concentrations in the soil (see Table 2) and therefore, it does not appear to be a useful indicator of the biological effects of metals on bacteria in contaminated soils. It is known that factors other than metal concentrations, including soil temperature, pH, the amount of organic matter (Richaume *et al*. 1989; van Elsas and Trevors 1990) and soil heterogeneity (Trevors *et al*. 1987), affect the transfer of genetically mobile elements. Nevertheless, it is interesting that genes conferring resistance to Cu, like those conferring resistance to Hg, are present in agricultural soils. The indication that transfer frequency may be higher in Cu-contaminated soils demonstrates a potential use in future studies on environmental gene transfer.

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