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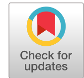
Mobile genetic elements and antibiotic resistance in mine soil amended with organic wastes



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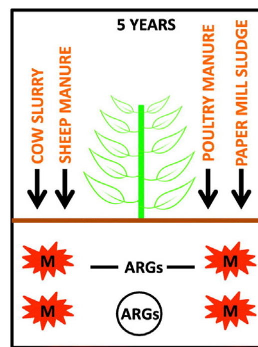
HIGHLIGHTS

- Mine soil can be a reservoir of mobile genetic elements.
- Almost all putative *E. coli* transconjugants displayed a multi-resistant phenotype.
- One putative *E. coli* transconjugant harboured imipenem resistance.
- Putative *E. coli* transconjugants displayed an altered fitness.
- Genome sequencing revealed no additional genes in the putative transconjugants.

GRAPHICAL ABSTRACT



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ABSTRACT

Metal resistance has been associated with antibiotic resistance due to co- or cross-resistance mechanisms. Here, metal contaminated mine soil treated with organic wastes was screened for the presence of mobile genetic elements (MGEs). The occurrence of conjugative IncP-1 and mobilizable IncQ plasmids, as well as of class 1 integrons, was confirmed by PCR and Southern blot hybridization, suggesting that bacteria from these soils have gene-mobilizing capacity with implications for the dissemination of resistance factors. Moreover, exogenous isolation of MGEs from the soil bacterial community was attempted under antibiotic selection pressure by using *Escherichia coli* as recipient. Seventeen putative transconjugants were identified based on increased antibiotic resistance. Metabolic traits and metal resistance of putative transconjugants were investigated, and whole genome sequencing was carried out for two of them. Most putative transconjugants displayed a multi-resistant phenotype for a broad spectrum of antibiotics. They also displayed changes regarding the ability to metabolise different carbon sources, RNA: DNA ratio, growth rate and biofilm formation. Genome sequencing of putative transconjugants failed to detect genes acquired by horizontal gene transfer, but instead revealed a number of nonsense mutations, including in *ubiH*, whose inactivation was linked to the observed resistance to aminoglycosides. Our results confirm that mine soils contain MGEs encoding antibiotic resistance. Moreover, they point out the role of spontaneous mutations in achieving low-level antibiotic resistance in a short time, which was associated with a trade-off in the capability to metabolise specific carbon sources.

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1. Introduction

In the soil ecosystem, the proximity of bacterial cells within microniches or biofilms favours the exchange of genetic determinants via horizontal gene transfer (HGT) (Christensen et al., 1998). Due to the administration of antibiotics to livestock, animal manure is often considered as a reservoir of bacteria carrying transferable antibiotic resistance genes (ARGs). The use of manure as organic fertilizer may increase HGT and transfer of ARG in environmental habitats (Götz and Smalla, 1997). Furthermore, high metal concentrations in soil can negatively affect soil microbial communities (Epelde et al., 2010); they can select for genes encoding metal resistance, which in turn can be associated with antibiotic resistance due to co-resistance (different resistance determinants or ARGs on the same genetic element) or cross-resistance (same genetic determinant for resistance to both antibiotics and metals) mechanisms (Seiler and Berendonk, 2012).

Although acquisition of MGEs can provide bacterial hosts with an array of beneficial traits such as antibiotic or metal resistance (Heuer and Smalla, 2012), it can also result in metabolic costs and changes in ecological fitness of recipient bacteria, owing to the replication, transcription and translation of acquired genes (Martínez et al., 2009). As consequence of these fitness costs, in the absence of selection pressure for their maintenance, MGEs can be lost from the bacterial population or the proportion of cells carrying MGEs can decrease (Jechalke et al., 2013). Alternatively, recipient bacteria can adapt through mutations on the chromosome or on the MGE itself, or by integration of the beneficial determinants into the chromosome (Dahlberg and Chao, 2003; Dionisio et al., 2005). Thus, fitness costs play a crucial role in the evolutionary dynamics of resistance, as they generate selection against such resistance (Zur Wiesch et al., 2011).

The aim of this work was to search for MGEs and ARGs in metal-contaminated mine soil treated 5 years before with manure-based fertilizers. To our knowledge, only very few studies have investigated the long-term effects of different organic amendments on the presence of MGEs and ARGs in metal contaminated mine soil. Besides, we studied the ability of an *E. coli* lab strain to acquire MGEs and ARGs from the soil bacterial community under antibiotic selection pressure. We hypothesized that, even five years after treatment, organically amended mine soil would (i) show a higher abundance of MGEs, (ii) trigger HGT of ARGs and metal resistance more efficiently than untreated soil and (iii) that acquired antibiotic resistance by recipient strains is an indication for successful conjugative transfer.

2. Materials and methods

2.1. Experimental design

The study area (43°13′ N, 3°26′ W) is located in an abandoned mine (Spain) (Barrutia et al., 2011). Within the mining area, two sites showing different levels of metal contamination (Cd, Pb, Zn) were selected (site 1 being less and site 2 being more contaminated; Table 1). From both sites, samples were collected from three randomly spatially distributed 1 m^2 plots, where the following amendments had been applied five years before, as part of an aided phytostabilisation field trial (for more information, see Galende et al., 2014 and Garaiyurrebaso et al., 2017): cow slurry (COW), sheep manure (SHEEP), poultry manure (POULTRY), and paper mill sludge mixed with poultry manure (2:1, v/v) (PAPER). For both sites, controls, where no amendment was added, were included.

Composite samples (10 sub-samples; 0–10 cm depth) were randomly collected from treated and control soils. Samples were kept at 4 °C during transport to the laboratory. Prior to DNA extraction and determination of physicochemical properties, the samples were sieved with a sterile sieve (2 mm) and stored at 4 °C for less than one week.

Table 1
Soil physicochemical properties based on dry weight (mean ± SE).

	SITE 1	SITE 2
Classification	Sandy-loam	Loam
pH (1:2.5 w/v, water)	6.49 ± 0.11	6.71 ± 0.03
Total organic matter (g kg^{-1})	199.8 ± 28.3	151.2 ± 9.0
Nitrogen total (g kg^{-1})	7.13 ± 0.87	4.27 ± 0.54
Phosphorus (Olsen) (g kg^{-1})	0.007 ± 0.001	0.002 ± 0.000
Calcium (g kg^{-1})	1.34 ± 0.09	0.76 ± 0.08
Magnesium (g kg^{-1})	0.29 ± 0.03	0.18 ± 0.01
Potassium (g kg^{-1})	0.16 ± 0.03	0.08 ± 0.00
Cation exchange capacity (cmol kg^{-1})	19.98 ± 2.83	15.12 ± 0.90
Cadmium (Cd) (mg kg^{-1})	6.00 ± 0.31	13.60 ± 0.16
Lead (Pb) (mg kg^{-1})	16,285 ± 188	28,587 ± 330
Zinc (Zn) (mg kg^{-1})	15,529 ± 179	61,007 ± 352

2.2. MGEs in soil samples

Total community DNA was extracted from 0.5 g of soil using the Fast DNA™ SPIN kit (MP Biomedicals, USA) and purified by Geneclean Spin Kit (MP Biomedicals, USA). Polymerase chain reaction (PCR) and Southern blot DNA hybridisation were used to study the presence of (1) class 1 integron integrase (*intI1*) and quaternary ammonium compound resistance (*qacEΔ1*) genes, (2) plasmid backbone regions related to replication (*trfA* of IncP-1 subgroups, IncN *rep* and V216 *rep*) and (3) origins of replication (IncQ *oriV*). Amplification of *intI1* and *qacEΔ1* genes was performed as described by Sandvang et al. (1997); of *trfA* gene from IncP-1 subgroups, as described by Bahl et al. (2009); of IncQ *oriV* and IncN *rep*, as described by Götz et al. (1996); and of pV216 *rep*, as described by Heuer et al. (2009). Oligonucleotide sequences, reference plasmids (positive controls), annealing temperatures and amplicon sizes are listed in Supplementary Table S1.

Southern blot DNA hybridisation was performed for all target genes, regardless of the detection of PCR products. Blotting and subsequent chemiluminescence detection of digoxigenin (DIG)-labelled DNA hybrids was performed according to the DIG System User's Guide for Filter Hybridisation (Roche, Germany). DIG-labelled probes were generated from PCR-amplified fragments obtained with the reference plasmids (Supplementary Table S1). PCR products (75 µl each) were separated by agarose gel electrophoresis in 1× TBE buffer at 50 V for 4–5 h. DNA was then denatured and transferred to a Hybond-N^+ membrane. Detection was carried out with CDP star® ready to use solution (Roche, Germany). The membrane was exposed to high performance chemiluminescence film (GE Healthcare Life Science, UK) and then developed.

2.3. Capturing transferable antibiotic resistance genes from soil bacteria

The exogenous plasmid isolation technique was used to capture transferable resistances into rifampicin resistant *E. coli* 1030. Initially, bacteria were detached from the soil matrix by mixing 10 mg of soil and 1.5 ml of 7.5 mM sodium pyrophosphate and Tween 80 (0.5%, w/v). Samples were shaken for 45 min at room temperature. The mixture was then allowed to settle for 5 min and the supernatant with detached bacteria served as donor. Recipient cells (*E. coli* 1030, RIF^R) were grown overnight at 37 °C in 2 ml TSB medium supplemented with rifampicin (100 µg ml^{-1}). One mililiter of the recipient culture, as well as 1 ml of donor solution were centrifuged at 4850 ×g at room temperature. Both recipient and donor supernatants were then discarded, pellets washed twice in 1 ml sterile 1/10 TSB solution and resuspended in 1 ml sterile 1/10 TSB. Subsequently, 500 µl each of recipient and donor were mixed and centrifuged at 4850 ×g at room temperature. Pellets were resuspended in 50 µl sterile 1/10 TSB and applied to a nitrocellulose filter (0.22 µm pore size; Millipore, USA). Filters were incubated overnight at 28 °C on TSA plates supplemented with cycloheximide (300 µg ml^{-1}). The bacterial lawn was resuspended in 1 ml sterile NaCl solution (0.85%, w/v) and stirred at 450 rpm for 20 min, to release bacterial

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