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Characterization of a collection of plasmid-containing bacteria isolated from an on-farm biopurification system used for pesticide removal

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ABSTRACT

Biopurification systems (BPS) are complex soil-related and artificially-generated environments usually designed for the removal of toxic compounds from contaminated wastewaters. The present study has been conducted to isolate and characterize a collection of cultivable plasmid-carrying bacterial isolates recovered from a BPS established for the decontamination of wastewater generated in a farmyard.

Out of 1400 isolates, a collection of 75 plasmid-containing bacteria was obtained, of which 35 representative isolates comprising in total at least 50 plasmids were chosen for further characterization. Bacterial hosts were taxonomically assigned by 16S ribosomal RNA gene sequencing and phenotypically characterized according to their ability to grow in presence of different antibiotics and heavy metals. The study demonstrated that a high proportion of the isolates was tolerant to antibiotics and/or heavy metals, highlighting the on-farm BPS enrichment in such genetic traits. Several plasmids conferring such resistances in the bacterial collection were detected to be either mobilizable or selftransmissible. Occurrence of broad host range plasmids of the incompatibility groups IncP, IncQ, IncN and IncW was examined with positive results only for the first group. Presence of the IS*1071* insertion sequence, frequently associated with xenobiotics degradation genes, was detected in DNA obtained from 24 of these isolates, strongly suggesting the presence of yet-hidden catabolic activities in the collection of isolates. The results showed a remarkable diversity in the plasmid mobilome of cultivable bacteria in the BPS with the presence of abundant resistance markers of different types, thus providing a suitable environment to investigate the genetic structure of the mobile genetic pool in a model on-farm biofilter for wastewater decontamination in intensive agricultural production.

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1. Introduction

In recent years, a great interest has arisen in the study of microbial communities in contaminated environments since their associated microbiota represents a reservoir for diverse types of resistance determinants as well as catabolic genes. Several polluted environments have been chosen as target sites for searching genes for organic-compounds degradation (Choi et al., 2009; Herrick et al., 1997; Zhang and Anderson, 2012), antibiotic resistance (Smalla et al., 2000) and heavy-metal resistance (Rajkumar et al., 2012; Smit et al., 1998) among others, and many of those loci have been demonstrated to be encoded on plasmids and other mobile genetic elements (MGE) (Anjum et al., 2012; Sharma and Thakur, 2009; Shin et al., 2012; Tabata et al., 2011).

In order to minimize agricultural contamination, on-farm biopurification systems (BPS) have been developed as a strategy for removing toxic compounds from polluted wastewater sources generated in the farmyard. BPS receive high loads of pesticides and in cases other toxic compounds at relatively rich concentrations during a substantial part of the year, thus imposing a strong, constant and long-term selective pressure on the evolution and growth of the associated microbiome (Sniegowski et al., 2011). For these reasons bacterial communities within BPS are research targets of practical and basic interest.

In the present study, we characterized a collection of plasmid-containing bacteria isolated from an on-farm BPS composed of a mixture of substrates such as cow manure, straw, willow chippings, soil, coconut chips, garden-waste compost and peat (De Wilde et al., 2010; Dunon et al., 2013).The list of all pesticides added to the BPS was reported in detail by Dealtry et al. (2014) and included at least fifteen different compounds.

The study of catabolic plasmids has been focused on the characterization of small and large plasmids that can be potentially used either in biotechnological assays or bioremediation strategies (Zhang et al., 2012). Particularly, we characterized community members from a BPS that was previously pointed as an environment with high abundance of IncP plasmids and other MGE (Dealtry et al., 2014; Gaze et al., 2013).

We present here the phenotypic and molecular characterization of a collection of plasmid-containing cultivable bacteria isolated from our model BPS. We characterized plasmid abundance and taxonomy of the bacterial hosts, plasmid size, their incompatibility groups, mobilization properties, and finally the presence of common traits frequently associated to plasmids and other MGE. Although the experimental strategy was limited to cultivable microorganisms, this *modus operandi* offered several advantages. First, the methodology allowed maintenance of plasmids in their natural hosts, enabling host-identification. Second, no plasmid-encoded selectable markers were required, which enabled the acquisition of a wider range of plasmid-containing bacteria, since many plasmids do not confer easily identifiable phenotypes on their host bacteria. The results that we present in this work show a remarkable diversity of cultivable plasmid-containing bacterial hosts in the biofilter, a significant proportion of transmissible plasmids, and several phenotypic characteristics that suggest the exposition of the BPS to other selective pressures (i.e. antibiotics, heavy metals) than the known high loads of pesticides.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli CV601 (Rf^r GFP) (Smalla et al., 2006) and *E. coli* CSH26 (Rf^r) (Miller, 1972) were used as recipient strains in bi- and triparental matings. *E. coli* HB101 carrying plasmid pRK600 (Finan et al., 1986) or pRK2013 (Figurski and Helinski, 1979) were used as helper strains in the triparental matings. *Ensifer* (*Sinorhizobium*) *meliloti* MVII-1 was used as plasmid molecular weight marker in *in situ* lysis gel electrophoresis (Kosier et al., 1993).

2.2. Biopurification system sampling

Samples were obtained from a pesticide-containing biofilter in Kortrijk, Belgium, containing a biomix composed of coco chips, straw, manure and field soil (Dunon et al., 2013). The BPS was used to treat water contaminated with different types of pesticides resulting from spillage and for residue water collected when cleaning the spraying equipment. Of the BPS mixture, 250 g were collected in March 2011 as was described by Dealtry et al. (2014) and stored for 2 weeks at 4 °C in the dark.

2.3. Recovery of bacteria from a BPS

Plasmid-containing bacteria were isolated as follows: 5 g of BPS sample was resuspended in 50 ml of sterile physiological-saline solution and the suspension was shaken at 180 rpm for 1 h at room temperature. The bacteria were harvested from the supernatant and aliquots plated in different cycloheximide-containing media (200 μg/ml) as well as in antibiotic-containing Luria-Bertani (LB) medium (Sambrook et al., 1989) and incubated at 28 °C for 48 h. Eight different types of agar plates were used for colony isolation: LB, tryptone-yeast (TY; Beringer, 1974), eosin–methylene-blue (EMB; Levine, 1918), MacConkey (Mck; Macconkey, 1905), dextrose-agar (Dex; Sigma-Aldrich), Mueller-Hinton (MH; Oxoid Ltd, UK), glutamate-sucrose minimal medium (GS) (Del Papa et al., 1999) and M9 minimal medium (M9) (Kempner and Miller, 1972). When required, the following antibiotics were added to LB medium: 120 μg/ml neomycin (Nm), 400 μg/ml streptomycin (Str), 200 μg/ml ampicillin (Ap), 10 μg/ml tetracycline (Tc), 25 μg/ml kanamycin (Km), 50 μg/ml gentamicin (Gm), 20 μg/ml trimethoprim (Tp), 200 μg/ml erythromycin (Er), 20 μg/ml chloramphenicol (Cm), 5 μg/ml nalidixic acid (Nx), 50 μg/ml carbenicillin (Cb) or 100 μg/ml rifampicin (Rf).

2.4. Plasmid screening

Single colonies obtained from plating on different media were grown in LB liquid medium overnight at 28 °C and

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180 rpm. In order to select those isolates that harbored one or more plasmids, the *in situ* lysis gel assay described by Eckhardt (1978) was used with the modifications described by Pistorio et al. (2008).

2.5. Preparation of plasmid DNA for evaluation of incompatibility groups

Isolates were grown on plates of R2A medium (two plates for each isolate). The freshly grown colonies were harvested in 2 ml $MgSO_4$ and centrifuged at 3000 × *g* for 3 min. The pellets were washed three times in 1 ml $MgSO_4$ then plasmid DNA was extracted as described by Smalla et al. (2000). The plasmid DNA quality was checked by agarose gel (0.8%, w/v) electrophoresis.

2.6. Southern blot hybridization

Plasmid DNA was digested with *Pst*1 and *BstZ*17I or *Not*1 enzymes (Fermentas GmbH, St. Leon Rot, Germany). The digested fragments were separated by electrophoresis on agarose gel (1.0%, w/v) and Southern blotted. Southern blot hybridization for the plasmid restriction digests obtained from *Pst*1 and *BstZ*17I enzymes was performed with IncP-1 mixed probes (α, β, γ, δ and ε subgroups), IncP-7, IncP-9 or IncQ probes. The IncP-1 mixed probe is a mixture of digoxigenin-labeled probes, obtained from PCR amplifications targeting different IncP-1 subgroups from reference plasmids (Appendix: Supplementary Table S1). The IncW and IncN probes were used in Southern blot hybridization for the plasmid restriction digests obtained from *Not*1 enzyme. Southern blot hybridization was performed according to Binh et al. (2008). The plasmids pKJK5, pCAR1, pWWO, RSF1010, RN3 and R388 were used as representative positive control for IncP-1, IncP-7, IncP-9, IncQ, IncN and IncW probes respectively. The primer systems, gene fragment and reference plasmids used to generate IncP-1, IncP-7, IncP-9, IncQ, IncN and IncW were listed in Appendix: Supplementary Table S1 (Bahl et al., 2009; Binh et al., 2008; Götz et al., 1996; Heuer and Smalla, 2007; Moura et al., 2010).

2.7. Amplifications by PCR

2.7.1. 16S rDNA PCR

Amplification of the 16S rDNA was performed with the universal bacterial primers 27f and 1385r (Weidner et al., 1996). PCR amplifications were carried out containing the PCR reaction buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.0) plus 200 μM each of the dNTPs, 3 mM $MgCl_2$, 2 μl of DNA template and 1U of T-free polymerase (Inbio-Highway, Argentina). The 16S rRNA gene PCR products were sequenced at the sequencing service from INTA Castelar, Argentina.

2.7.2. *IS*1071 *PCR*

Amplification of a 179-bp *tnp*A fragment of insertion sequence IS*1071* was performed with primers IS-F and IS-R as described by Providenti et al. (2006). Plasmid pMS025, containing a single IS*1071* copy originatied from the IncP-1 plasmid pUO1, was used as a positive control in *tnp*A-targeted conventional PCR. PCR was performed according to Dunon et al. (2013) and the PCR products were sequenced at INTA Castelar, Argentina.

2.8. Metal and antibiotic resistance assays

The minimum inhibitory concentrations (MICs) of the heavy metals for each representative isolate were determined by plating on media containing serial dilutions of each one. In this manner, tolerance to the following metals was investigated: Ni^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, and Hg^{2+} ($NiCl_2.6H_2O$, $CdCl_2$, $CuSO_4.5H_2O$, $MnCl_2.4H_2O$, $CoCl_2.6H_2O$, $ZnSO_4.7H_2O$, or $HgCl_2$). Filter-sterilized stock solutions of each metal were added to LB-agar medium at different final concentrations ranging from 2.5 μM to 20 mM. Exponential-phase cultures grown in LB were serially diluted (1:10) and 10 μl of a dilution containing 10^6 cfu/ml spotted onto the metal-containing LB plates and incubated at 28 °C for 48 h. After assessment of the resulting growth, the MIC was calculated for each isolate.

Representative isolates were tested for sensitivity to antimicrobial agents as follows: cultures were grown in LB medium at 28 °C and 180 rpm until they reach an $OD_{600} = 0.5$. Then they were serially diluted (1:10) and 10 μl of a dilution containing 10^6 cfu/ml inoculated onto LB plates containing the following antibiotics: Ap (6.25–1,600 μg/ml), Cm (0.625–160 μg/ml), Km (1.56–400 μg/ml), Nx (1.25–40 μg/ml), Nm (3.75–480 μg/ml), Rf (3.13–100 μg/ml), Sm (3.13–1,600 μg/ml), Gm (0.75–50 μg/ml), Tp (2.5–320 μg/ml), ciprofloxacin (8–250 ng/ml) or Tc (0.313–40 μg/ml). Plates were incubated at 28 °C for 48 hs.

2.9. Plasmid mobilization assay

Matings were carried out using *E. coli* strain CSH26 and CV601 as recipient for antibiotic- and heavy metal-based selection, respectively. All matings were performed using the HB101 (pRK600), except when the selection media contained chloramphenicol, in which case HB101 (pRK2013) was used. Matings were performed as follow: exponential-phase liquid cultures of the donors (BPS isolates), recipients (*E. coli* CV601 [Rf^r GFP] or *E. coli* CSH26 [Rf^r] strains) and helper strains (when required) were combined in equal quantities, mixed by gentle agitation and centrifuged at 800 × *g* for 8 min. The pelleted cells were resuspended in 50 μl of LB, spotted onto solid LB medium and incubated overnight at 28 °C. Transconjugants were selected after 48 h incubation at 28 °C on rifampicin-containing LB plates supplemented with different antibiotics or metals. The antibiotic and metal concentrations used for the selection of transconjugants were: 120 μg/ml Nm, 400 μg/ml Str, 200 μg/ml Ap, 10 μg/ml Tc, 50 μg/ml Km, 10 μg/ml Gm, 20 μg/ml Tp, 200 μg/ml Er, 20 μg/ml Cm, 5 μg/ml Nx, 50 μg/ml Cb, 200 μg/ml Rf, 5 mM Cd^{2+}, 7 mM Cu^{2+}, 3 mM Co^{2+}, 40 mM Mn^{2+}, 5 mM Ni^{2+}, 3 mM Zn^{2+}, or 0.22 μM Hg^{2+}. Parental strains were controlled in each selective medium. The transconjugants were screened for the presence of plasmids by *in situ* lysis gel assay.

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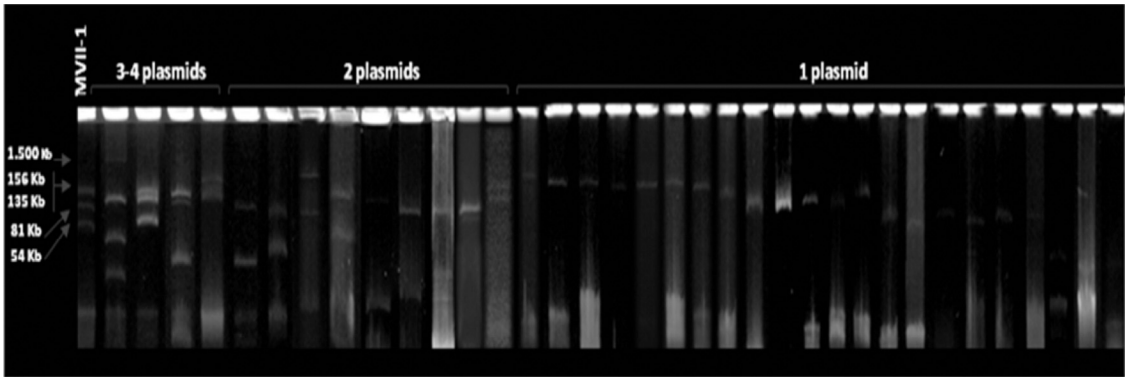

Fig. 1. Plasmid profiles from 35 representative isolates as analyzed by *in situ* gel lysis. Plasmid sizes were estimated from *S. meliloti* MVII-1 strain which carries five plasmids of known sizes (Hynes et al., 1986). Plasmids were detected ranging between 30 and 1500 kbp in length.

3. Results

3.1. Compilation of a collection of bacterial isolates harboring large plasmids from the BPS and their classification according to plasmid profiles

More than 1400 randomly selected bacterial isolates obtained from the biofilter suspension (see section 2.2) were screened by the *in situ* lysis assay to investigate their plasmid content (section 2.4). Out of the total isolates analyzed, 75 were identified to carry plasmids (5%). The *in situ* lysis technique allowed the classification of the isolates in 35 representative groups, according to their plasmid profiles (i.e., plasmid number and plasmid size) (Fig. 1). The whole collection comprises at least 50 plasmids ranging from 30 to 1500 kbp in size, with more than one-half being larger than 100 kbp. The method proved to be efficient in identifying plasmids within a broad range of sizes. The fact that plasmid profile diversity did not increase when the number of isolates analyzed was increased from 35 to 75 was indicative that the 35 plasmid profiles obtained were probably representative of the collection as a whole. The isolation medium for each representative isolate is provided in Appendix: Supplementary Table S2.

3.2. Taxonomic classification of the plasmid-containing isolates based on 16S rDNA analysis

Comparison of the 16S rDNA sequences obtained with those available in the GenBank database at National Center for Biotechnology Information (NCBI) indicated that the 35 isolates comprise a remarkable bacterial diversity (Appendix: Supplementary Table S2). The results revealed that out of 35 representative bacterial isolates that were analyzed, 22 corresponded to Gram-negative and 13 to Gram-positive. Fig. 2 shows the isolates number of the different classes and genera represented in the plasmid-containing BPS bacterial collection. The class Gammaproteobacteria, dominated by *Pseudomonas*, was the most highly represented (40%), followed by Actinobacteria (20%), Bacilli (17%), Betaproteobacteria (9%), Alphaproteobacteria (8%) and Sphingobacteria (6%).

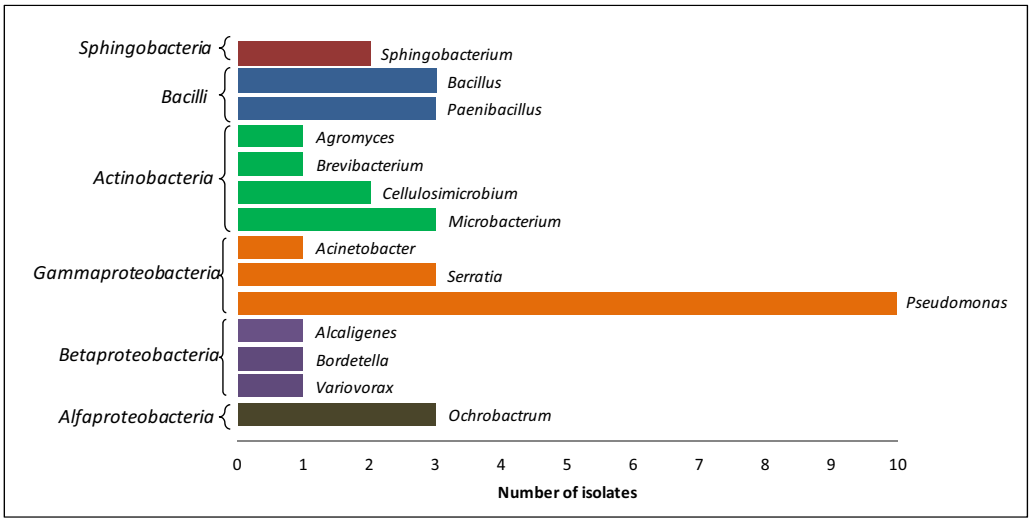

Fig. 2. Taxonomic classification of the 35 representative isolates.

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3.3. Antibiotic resistances and tolerance to heavy metals in the plasmid-containing isolates

The highest MICs for heavy metals determined for the isolates collection by the serial dilution method were: 40 mM for Mn^{2+}, 2 mM for Co^{2+}, 40 mM for Zn^{2+}, 2 mM for Cd^{2+} and 0.2 μg/ml for Hg^{2+} (Table 1). For Cu^{2+} and Ni^{2+}, the MIC was 2 mM, and all the isolates exhibited the same tolerance (data not shown). In addition, all except two isolates had a MIC of 0.2 mM for Co^{2+} (Table 1). Representative isolates were also tested to evaluate their tolerance to several antibiotics by the same serial-dilution method. Broad ranges of tolerance were observed for most antibiotics. As it is shown in Table 1, the maximum MICs for the different antibiotics were: 320 μg/ml for Tp, 1600 μg/ml for Ap, 40 μg/ml for Nx, 100 μg/ml for Rf, 0.5 μg/ml for ciprofloxacin, 1600 μg/ml for Sm, 480 μg/ml for Nm, 40 μg/ml for Tc, 400 μg/ml for Km, 200 μg/ml for Gm and 160 μg/ml for Cm. Most of the isolates showed tolerance to more than one antibiotic, thus displaying multiresistance phenotypes in different bacterial genera.

3.4. Analysis of the presence of BHR plasmids and the IS1071 element in the collection of representative isolates

Occurrence of plasmids belonging to the IncP, IncQ, IncW and IncN incompatibility groups was tested for the 22 Gram-negative isolates, where only two of them gave positive hybridization signals, one for the IncP-1 (*Alcaligenes sp.* BF42) and one for IncP-7 (*Pseudomonas putida* BF25) subgroups. The remaining isolates did not show positive hybridization signal for any of the other tested incompatibility groups, indicating that other types of plasmids are present in the BPS collection.

In order to determine if the mobile genetic element IS*1071* was present in our isolates collection, we looked for conserved sequences of its associated gene, *tnp*A. Of the analyzed isolates, 72% featured a strong positive amplification signal for the TnpA transposase from the IS*1071* (Appendix: Supplementary Table S3). Five of the PCR products were randomly chosen for sequence analysis. Their sequences showed a 93 to 100% nucleotide sequence identity with the IS*1071 tnpA* sequence confirming the specificity of the screening used.

Table 1
Minimum inhibitory concentrations (MICs) of heavy metals and antibiotics for the growth of representative bacterial isolates from the BPS.

Isolate	$MnCl_2.4H_2O$[a]	$CoCl_2.6\ H_2O$[a]	$ZnSO.7\ H_2O$[a]	$CdCl_2$[a]	$HgCl_2$[a]	Tp[b]	Ap[b]	Nx[b]	Rf[b]	Cp[b]	Sm[b]	Nm[b]	Tc[b]	Km[b]	Gm[b]	Cm[b]
BF02	2	0.2	2	0.2	0.01	320	1600	nd	25	0.062	1600	60	5	400	6	80
BF13	2	0.2	2	0.2	0.01	320	800	2.5	25	0.062	1600	60	5	400	6	80
BF14	2	0.2	2	0.02	0.005	nd	200	40	25	0.25	6.25	7.5	28	400	6	40
BF15	2	0.2	2	0.2	0.01	320	1600	5	25	0.25	1600	30	5	200	3	160
BF19	0.2	2	0.2	2	0.2	40	800	5	25	0.062	1600	nd	nd	nd	6	2.5
BF21	20	0.2	2	0.2	0.01	5	nd	nd	6.25	0.016	1600	nd	nd	3.12	nd	10
BF22	2	0.2	2	0.02	0.01	160	nd	2.5	6.25	0.016	1600	30	nd	6	nd	10
BF25	2	0.2	2	0.2	0.2	320	800	5	50	nd	1600	15	5	3.12	3	160
BF27	2	0.2	40	2	0.2	320	800	10	50	0.031	1600	15	5	3.12	3	40
BF28	2	0.2	2	0.02	0.01	10	nd	nd	nd	nd	25	nd	20	3.12	nd	10
BF30	2	0.2	2	0.2	0.01	20	25	40	25	0.25	25	60	0.625	400	3	2.5
BF31	2	0.2	0.2	0.02	0.01	40	25	40	25	0.25	12.5	7.5	2.5	200	50	10
BF33	20	0.2	2	0.2	0.02	320	400	20	25	0.016	25	15	0.625	3.12	1.5	80
BF36	2	0.2	0.2	2	0.01	10	nd	40	25	0.125	12.5	nd	5	25	3	1.25
BF37	20	0.2	2	2	0.01	40	25	1.25	25	nd	25	30	5	6	3	10
BF42	2	0.2	0.2	0.2	0.1	160	nd	2.5	6.25	0.031	400	nd	2.5	nd	1.5	2.5
BF43	20	0.2	2	2	0.02	20	25	2.5	50	0.016	12.5	7.5	5	6	3	10
BF44	2	0.2	2	0.2	0.01	nd	12.5	40	nd	0.25	25	7.46	40	3.12	3	10
BF46	40	2	2	0.2	0.01	5	400	2.5	100	0.031	12.5	30	40	3.12	3	10
BF48	2	0.2	2	0.02	0.01	320	nd	40	nd	0.125	25	nd	5	3.12	3	1.25
BF52	2	0.2	2	0.02	0.01	320	nd	40	nd	0.5	25	7.5	nd	3.12	6	1.25
BF53	0.2	0.2	0.2	0.01	0.01	nd	nd	20	6.25	0.25	12.5	nd	0.625	3.12	1.5	1.25
BF55	2	0.2	2	0.02	0.02	320	25	5	50	0.016	25	7.5	2.5	3.12	1.5	80
BF56	2	0.2	0.2	0.02	0.02	160	nd	2.5	6.25	0.016	12.5	7.5	0.625	6	1.5	10
BF58	2	0.2	2	0.2	0.02	320	1600	20	50	0.016	25	7.5	nd	3.12	1.5	160
BF59	2	0.2	2	0.2	0.01	80	800	20	25	0.25	800	480	20	400	200	10
BF60	2	0.2	2	0.2	0.01	320	800	5	nd	0.25	800	480	20	400	200	10
BF61	2	0.2	2	0.2	0.01	320	1600	5	50	0.062	25	15	5	3.12	1.5	80
BF62	2	0.2	2	0.2	0.01	320	1600	5	50	0.031	12.5	15	5	3.12	1.5	80
BF67	2	0.2	0.2	0.2	0.01	40	25	5	25	nd	25	15	0.625	6	3	10
BF68	2	0.2	0.2	0.2	0.01	40	25	nd	nd	nd	25	15	0.625	6	3	10
BF70	20	0.2	2	0.02	0.02	nd	nd	2.5	nd	0.016	12.5	nd	nd	3.12	nd	10
BF71	20	0.2	0.02	0.01	0.02	10	nd	nd	nd	nd	6.25	nd	nd	nd	3	1.25
BF73	2	0.2	0.2	0.01	0.005	10	nd	5	nd	0.016	6.5	nd	nd	6	3	1.25
BF75	2	0.2	2	0.02	0.02	40	25	40	6.25	0.031	25	7.5	0.625	25	3	10

[a] The numbers shown represent the lowest concentration (mM) of the particular metal that completely inhibits growth of the organism in LB-agar medium.
[b] The numbers shown represent the lowest concentration (μg/ml) of the antimicrobial agent that completely inhibits growth of the organism in LB-agar medium.
nd = not determined.

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Table 2
Overview of transmissible and conjugative plasmids found in the BPS isolates collection.

Donor isolate	Tri-parental mating	Bi-parental mating	Antibiotic/metal resistance	*tnp*A PCR in transconjugant
Cellulosimicrobium cellulans BF14	+	–	streptomycin, gentamycin	–
Microbacterium sp. BF36	+	–	streptomycin	+
Alcaligenes sp. BF42	+	–	tetracycline	+
Alcaligenes sp. BF42	+	+	Hg^{2+}	+
Pseudomonas sp. BF61	+	–	nalidixic acid	+
Bacillus sp. BF71	+	–	Zn^{2+}	–
Pseudomonas sp. BF75	+	–	Zn^{2+}	–

3.5. Mobilization properties of plasmid replicons present in the collection of culturable isolates from the BPS

Isolates belonging to the different groups defined by their plasmid profiles were assayed to investigate their ability to transfer/mobilize their plasmid/s. To do this, we used plasmid-free *E. coli* recipients along with representative strains as donors in bi- and tri-parental matings (in this last case using also a helper strain) (see section 2.9). In bi-parental matings, transconjugants were obtained when *Alcaligenes sp.* BF42 was used as donor strain and selection was performed in Hg^{2+} supplemented medium. Isolate *Alcaligenes sp.* BF42 also showed to contain a mobilizable plasmid carrying resistance to tetracycline, as could be deduced from the triparental mating. In this strain, mercury and tetracycline resistance genes are present in different plasmids since the tolerance to each of these chemicals was independently transferred (Table 2).

In addition, in triparental matings, mobilizable plasmids could be identified in 6 isolates out of the 35 isolates analyzed from the BPS collection (20%). These results indicate the presence of antibiotic and metal resistance genes in the mobilizable plasmids.

Interestingly, transconjugants were obtained when *Cellulosimicrobium cellulans* BF14, *Microbacterium sp.* BF36, *Bacillus sp.* BF71 were used as donors. The mobilized plasmids confer resistance to Gm/Sm, Sm and Zn^{2+} respectively. Results obtained indicate that these Gram-positive isolates carry plasmids with the ability to replicate in both Gram-positive and Gram-negative hosts (Table 2).

Finally, when the presence of the IS*1071* insertion sequence was analyzed in the *E. coli* transconjugants obtained, 4 of them were found to be positive (Table 2), thus indicating that this element was carried by the plasmids of the donor isolates.

4. Discussion

The analysis of MGE and HGT in soil bacteria is a critical issue for improving our current understanding of the mechanisms underlying microbial-genome dynamics. Within this context, the study of transmissible plasmids in the BPS bacterial community could help to describe the structure of the corresponding plasmid mobilome which could be considered one of the principal drivers of bacterial adaptation and diversity.

Plasmids capable of replication in a wide range of hosts are responsible for dissemination of genetic information between bacteria, even between different genera. Among the best characterized and described BHR plasmids are IncP (Dennis, 2005; Thomas and Smith, 1987), IncQ (Loftie-Eaton and Rawlings, 2012; Rawlings and Tietze, 2001), IncW (Fernández-López et al., 2006) and IncN (Carattoli et al., 2010; Zong et al., 2011). BHR plasmids are known to transfer genes between diverse groups of bacteria. Plasmids can provide their host a large array of phenotypes, such as antibiotics and heavy metals resistance, and also enable their hosts to degrade pollutants. Although some BHR plasmids have been extensively studied, their genetic diversity still remained largely unknown.

The goal of this study was to analyze selected plasmid-containing bacteria obtained from an on-farm BPS. Analysis of the plasmid content for a collection of culturable bacterial isolates by *in situ* lysis gel electrophoresis indicated that large-sized plasmids are present in the biofilter collection. Classification of 75 isolates allowed for the distinction of 35 representative diversity groups based on their plasmid profiles. Of the four BHR groups of plasmids analyzed, IncP1- and IncP7-like plasmids were detected in our collection. IncP-like plasmids are perhaps the most important group since they usually harbor genes involved in catabolic pathways (Dennis, 2005). In particular, IncP-1 plasmids are relatively large (50 to 500 kbp) and usually contain other MGE, such as integrons, insertion sequences, and transposons, carrying antibiotic and metal resistance genes as well as catabolic sequence clusters (Dennis, 2005). IncP-7 plasmids have been reported to be associated to non man-made pollutants. Two IncP-like plasmids were detected in our collection. Identification of IncP-1 and IncP-7 plasmids and mobilization assays showed that mobilizable and self-transmissible plasmids are present in the isolates. The low proportion of IncP-like plasmids within the bacterial collection suggested that plasmids of other incompatibility groups are responsible for the dissemination of information in the BPS community, as observed in the bi- and tri-parental mating assays. Moreover, it is conceivable that these plasmids encode replication and maintenance genes different than those from plasmids previously characterized in clinical and environmental isolates.

The presence of *tnp*A, as genetic marker for IS*1071,* was examined. The IS*1071* element has been found frequently flanking genes involved in the degradation of xenobiotics and other toxic organic compounds. IS*1071* is often incorporated as an insert into the backbone of plasmids, mainly in IncP-1, as well as in the chromosome of both Gram-negative and Gram-positive bacteria (Devers et al., 2007; Kim et al., 2013; Liang et al., 2005; Ma et al., 2007; Schlüter et al.,

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2007; Top and Springael, 2003; Vedler et al., 2004). Dunon et al. (2013) previously reported the presence of both IncP-1 and IS*1071* sequences in an environmental sample obtained from the same on-farm BPS used in this study. In our collection, 2 representative isolates were positive for both IS*1071* and IncP, indicating that in our collection IS*1071* elements can also be present in either the bacterial chromosomes or in plasmids that belong to other incompatibility groups. The collected information will be valuable to investigate for the presence of potential degradative gene clusters in the neighborhood of the identified IS*1071* elements, and to evaluate if any of them is associated to the activities of pesticides mineralization previously reported for the BPS under analysis (Dunon et al., 2013).

In our study, representative isolates were tested for their ability to grow in antibiotic and metal containing media. The MICs for antibiotics were highly variable for the different isolates, even within the same genera or species. This variability is exemplified by the *Pseudomonas putida* isolates BF25, BF33 and BF55 exhibiting differential growth capabilities in chloramphenicol, streptomycin and ampicillin containing media. This observation suggests that plasmids or other MGE could be responsible for these resistances. Plasmid mobility assays performed in the present work indicated that different isolates contained mobilizable plasmids carrying antibiotic resistance in consonance with the observation that most frequent mechanisms for transfer of antibiotic resistance genes among bacteria are mediated by conjugation *via* plasmids and/or genetic integrative elements (Perry and Wright, 2013). Since for the majority of the plasmids analyzed we could not assume the presence of any selectable marker, the observation that 20% of the matings generated transconjugants (Table 2) indicates that the proportion of plasmids that can be mobilized is probably higher than that value. Besides, the possibility should be considered that other plasmids could be transferred to the recipient strain at a very low frequency or has not the ability to replicate in the *E. coli* recipient strain.

All isolates showed a similar tolerance for Cu^{2+} and Ni^{2+}. However, marked differences were observed for the resistances to other metals such as Zn^{2+}, Cd^{2+}, Mn^{2+}, $Co^{2+}$and Hg^{2+}, even between isolates representing the same genera and species. Mobilization assays also evidenced the presence of metal resistance genes in the BPS plasmid collection. Multiple genes encoding antibiotic and metal resistances are frequently found in a same plasmid or MGE. Furthermore, independent loci may additively contribute to the resistance levels observed (Karbasizaed et al., 2003; Mergeay et al., 1985). It has to be considered that microbial exposure to one toxicant could also result in an indirect selection for bacteria with resistance to multiple, but chemically unrelated toxicants and/or antibiotics, possibly as a result of a corresponding HGT of MGE (Baker-Austin et al., 2006; Seiler and Berendonk, 2012).

Finally, it is noteworthy to mention that in agreement with our experimental results, Dunon et al. (2013) and Dealtry et al. (2014) confirmed a high abundance and diversity of MGE in samples from the same BPS, suggesting that this habitat can be considered a site of bacterial microevolution, and a suitable source for the isolation of genes encoding novel catabolic enzymes and resistances to toxic elements/compounds. The collection of isolates that we characterized here – which bear more than fifty high molecular weight plasmids (accounting for several megabases in length) – provide a valuable germplasm to mine for novel activities of interest, to characterize dissemination mechanisms of antibiotic, metal and pesticide resistances, and to investigate the genetic structure of the mobile genetic pool in a model environment of agricultural interest.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.plasmid.2015.05.001.

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