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PCR primers for detection and characterisation of IncP-9 plasmids

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Abstract

IncP-9 plasmids are best known as the vehicles for spreading biodegradation functions among *Pseudomonas* species but can also carry resistance determinants. New PCR primer systems targeting different replicon-specific regions were designed to allow detection of IncP-9 plasmids. Their specificity was checked against a range of IncP-9 plasmids as well as representatives of incompatibility groups IncFI, IncFII, IncN, IncQ, IncP-1α, IncP-1β, IncP-2, IncP-7, IncP-13, IncW, IncU, IncX and IncZ. Products obtained for plasmids assigned to IncP-9 group by traditional incompatibility testing varied in size and restriction pattern suggesting diversity in the 'core' sequence among related replicons. Specific primer pairs were applied to community DNA extracted from a range of environments including those subject to strong selective pressure, caused by antibiotics, metals and organic pollutants. Abundant products were observed in manure and sewage, independently of the presence of antibiotics and metals, but could also be detected in coastal water and streptomycin-treated soil. Community DNA from faeces of piglets treated and non-treated with Zn gave particularly strong PCR product with IncP-9 *rep* primers. Therefore, an attempt was made to isolate bacteria carrying the IncP-9-like plasmids, but this was not successful. The results of application of these newly designed primer pairs to plasmid isolates as well as community DNA indicate that the IncP-9-related plasmids are a diverse family prevalent in various environments and widely distributed geographically.

Keywords: Polymerase chain reaction primer; Total community DNA; IncP-9 plasmid; Biosafety; Bioremediation; Horizontal transfer

1. Introduction

Plasmids are important vehicles in maintenance and spread of genes in bacteria. They contribute significantly to the diversity and adaptability of bacteria and have played a key role in the evolution of antibiotic resistance and biodegradation determinants in response to selective pressure created by man's activities [1]. They have also been exploited as the vehicles for gene cloning and genetic manipulation in bacteria. All these aspects create the need to monitor and track plasmids in medical, agricultural and industrial environments. Until recently this depended on screening cultured bacterial strains for plasmids by isolation and analysis of the plasmid DNA carried. However, PCR-based detection of plasmid-specific sequences in total community DNA, independently of the culturability of the bacterial host, now provides more reliable data on plasmid distribution [2]. The other advantage of this approach is that large sample numbers can be analysed, enabling extensive screening for a variety of environments. Primer systems for PCR amplification of replicon-specific DNA regions can be designed on the basis of available sequence data [2–4]. These primers have been used previously to analyse the presence of IncN, IncQ, IncP-1α, IncP-1β, IncP-9 and IncW plasmids in various soils and manure slurries, oil seed rape, potato rhizosphere samples with and without copper treatment, fish farm sediment, coastal salt marsh, sewage, compost and waste water [2,5]. A generalisation from these studies appeared to be that while plasmids of these groups are widely distributed, in many

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cases the strength of signal increased significantly when a specific environment was treated with a selective agent. Primers allowing amplification of IncP-1 (RK2) and IncFIC *oriV* as well as repSD and repGA sequences have also been applied to total community DNA from marine sediments [6–8].

Here we focused on the IncP-9 plasmids that appear to be common vehicles for degradative pathways as well as a range of resistance determinants. DNA sequence analysis and hybridisation experiments performed for members of the IncP-9 group revealed a high similarity of their putative replication regions [9,10]. Sequence conservation within a cluster of replication as well as stability and transfer genes between two representatives of the IncP-9 group, pM3 R-plasmid [11] and pWW0 D-plasmid [12], enabled us to further develop and use IncP-9 replicon-specific primer systems for PCR, plasmid group-specific probes and sequencing. We propose them as tools for assessing the diversity of the IncP-9 replication and stability region (the core of the IncP-9 backbone) and distribution of IncP-9 plasmids in environmental samples. To this end we chose representative samples from a range of habitats including some pairs of environments with and without selective agents associated with agricultural and industrial environments. They should thus be useful in investigating the distribution of IncP-9 plasmids and their role in adaptive responses of their bacterial host to selective pressure.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli K53 (*met pro*) was used as the standard host. Reference plasmids used were R386 (IncFI), R1 (IncFII), RA3 (IncU), R6K (IncX) [13], RN3 (IncN) [2], RP4 (IncP-1α) [14], R751 (IncP-1β) [2], pBS12 (IncP-2), pMG25 (IncP-13) [15], Rms148 (IncP-7) [16], pMT2 (IncP-9) [10], RSF1010 (IncQ) [17], R388 (IncW) [18] and R545 (IncZ) [19]. Representatives of the collection of degradative and resistance plasmids which have been placed in the IncP-9 group by classical incompatibility testing and examined in this study are presented in Table 1.

Bacteria were grown in Luria–Bertani (LB) broth cultures [20] at 28°C overnight or M9 minimal medium [21] with agar added (1.5% w/v) supplemented with methionine and proline and at least one antibiotic for which a resistance was encoded by the respective plasmid. Antibiotics and components that have been used for selection were added as follows: ampicillin (100 μg ml^{-1}), kanamycin sulfate (25 μg ml^{-1}), penicillin G (300 μg ml^{-1} in agar, 120 μg ml^{-1} in liquid medium), streptomycin sulfate (10–30 μg ml^{-1}), tetracycline hydrochloride (10–15 μg ml^{-1}), chloramphenicol (25 μg ml^{-1}), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal; 0.02%) and isopropyl β-D-thiogalactoside (IPTG; 1 mM).

2.2. Plasmid DNA isolation and manipulation

Large degradative plasmids were isolated on a large scale using the alkaline lysis method followed by the CsCl density gradient purification and removal of ethidium bromide [22]. A small scale according to the method of Birnboim and Doly [23] with modifications described by Smalla et al. [24] was applied to extract plasmids from culturable fractions of environmental bacteria. Reference plasmid extractions were performed from 5-ml overnight cultures with the Qiagen midi prep (from Qiagen, Hilden, Germany). DNA was manipulated and analysed using techniques described by Sambrook et al. [22] and enzymes provided by MBI Fermentas or Roche and New England Biolabs. DNA fragments were purified using the Geneclean kit (from Bio101) or the High Pure PCR Product Purification kit (from Roche Diagnostics, Mannheim, Germany). PCR fragments resulted from IncP-9-specific amplification of reference and putative IncP-9 plasmids as well as total DNA isolated from sewage coming from Zn-treated and untreated pigs were cloned into the vector pGEMT-Easy (from Promega, Southampton, UK) and transformed into *Escherichia coli* DH5α using the standard $CaCl_2$ transformation protocol [22]. Extraction of the clones was performed with the Promega Wizardprep (from Promega, Southampton, UK) to ensure purity of DNA crucial for sequencing.

2.3. Primers

Properties of oligonucleotide primers used in this study: their sequences, annealing temperatures and product sizes are summarised in Table 2. Basic primers specific for plasmid 'backbone' regions related to replication of IncP-1–*trfA2* [2], IncP-9 [4], IncN–*rep*, IncQ and IncW–*oriV* [2] were previously described. New primers designed on the basis of the IncP-9 'core' genes proposed to be involved in plasmid partitioning (*parA*, *parB*, *korA*, *tolA* and *res*) [10] as well as the extended replication region including *oriV* were designed using the Netprimer program (Premier Biosoft International).

2.4. PCR conditions

The Inc-specific PCR was performed with either heat-denatured cells or plasmid DNA as a template and also with total community DNA isolated from sewage, manure, soil, rhizosphere and water. With the exception of amplification of the two large fragments, PCR mixtures contained 5 U of *Taq* polymerase (Stoffel fragment; [25]), Stoffel buffer, 0.2 mM deoxynucleoside triphosphates, 3.75 mM $MgCl_2$, and 0.2 μM of each primer (0.4 μM each of IncP-9 primers). After a 5-min step at 94°C, 35 cycles of amplification consisting of 1 min of denaturation, 1 min of primer annealing at the appropriate annealing temperature (Table 2), and 1 min of primer

extension at 72°C were carried out, followed by a 10-min final extension step at 72°C. To amplify fragments flanked by pairs *mpfA*1Fa–*korA*2Ra and *korA*3Fa–*rep*3Rc, 4 U of Qiagen (Crawley, UK) *Taq* polymerase with *Taq* polymerase buffer supported by Q solution were used together with 0.2 mM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, and 0.4 μM of each primer. The reaction in all cases had the same scheme. The denaturing step of the reaction carried out at 94°C for 5 min was followed by 30 cycles of 94°C (1 min), an annealing step at the temperature appropriate for the pair of primers (1 min), 72°C (2.5 min), and a final extension step at 72°C (10 min). PCR products were analysed in 1% agarose gels with Tris–borate–EDTA buffer and Southern blotted (Fig. 1). Amplified fragments were hybridised with respective digoxigenin-labelled probes. Positive controls were RN3 for IncN, RP4 for IncP-1α, R751 for IncP-1β, pMT2 for IncP-9, RSF1010 for IncQ. Negative controls contained water instead of target DNA.

2.5. Restriction analysis of PCR products

RFLP analysis was performed on products of *mpfA*1Fa–*korA*2Ra and *korA*3Fa–*rep*3Rc amplification of different plasmids assigned to the IncP-9 group. Restriction digestions consisted of 10 μl of a PCR mixture in a total volume of 30 μl with 2–3 U of a single restriction enzyme provided by MBI Fermentas (Helena Biosciences, Sunderland, UK). Restriction mixtures were incubated at 37°C for 1.5–3 h. Digested products were subjected to electrophoresis on 1.6% agarose gel in 0.5×TBE buffer at 120 V. Banding patterns were visualised by ethidium bromide staining.

2.6. Generation of probes

PCR products obtained with the different primer systems with the respective reference plasmids were cut out of preparative gels, recovered with the Geneclean kit (Bio101; Anachem, Luton, UK) and digoxigenin labelled by random priming according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).

2.7. Southern blot hybridisation

Southern blots were made for analysis of all of the PCR products obtained using Inc-specific primer systems, according to the standard protocol described by Sambrook et al. [22]. The hybridisations were performed at 62°C following the instructions for the digoxigenin detection kit (from Roche). When it was necessary to reprobe membranes with a different probe, the previously bound labelled probe was removed according to the manufacturer's recommendations.

2.8. Extraction of total community DNA from environmental samples

Sewage samples were taken every 7 days from pigs fed with a standard diet or one supplemented with Zn (4.5 g ZnO per kg of feedstuff). Total community DNA was extracted from 0.3–0.5 g of sewage using four samples from seven independent samplings of each type (without or with Zn) as well as from composite samples (a mixture of manure from the animals fed with Zn and the controls). The extraction was performed with an Ultra Clean Soil DNA Isolation Kit (MoBio, Cambridge, UK) modified by replacing an initial vortexing step with a bead beating method (shaken in a Braun homogeniser two times for 30 s at the highest speed in Bead Solution tubes provided by MoBio). Other total community DNA samples tested in this study were extracted by the same procedure directly from soil, rhizosphere, manure, sewage and water, and were provided by participants of the EU-funded Project RESERVOIR (BIO4-CT580053) originated from different parts of Europe. Sources of these environmental samples are shown in Table 3 and further details can be obtained from Heuer et al. [26]. Amplification of a region of 16S rRNA gene using primers (F984GC and R1378) and subsequent DGGE [27,28] was used to ensure that community DNA was of sufficient purity for use in PCR assays for IncP-9 gene amplification. Only samples that gave a standard strength of signal were used for further PCR analysis.

2.9. Extraction of bacteria from pig sewage

One gram of pig sewage samples in 0.85% NaCl (4 ml) was treated in a stomacher laboratory blender (Seward Medical, Northampton, UK) before plating. The resulting suspensions were serially diluted and plated in duplicate on LB agar supplemented with cycloheximide (200 μg ml^{-1}) to suppress fungal growth on plates. After 2 days of incubation at 28°C mixed bacterial cultures from the plate were suspended in saline (0.85% w/v NaCl), harvested by centrifugation and washed by resuspension in saline and re-centrifugation. Pellets were used for small-scale plasmid DNA isolation as described above.

2.10. DNA sequencing

Sequencing of fragments obtained from IncP-9-specific PCR was performed using 'universal' primers for the flanking vector (pGEM-T) sequences and custom oligonucleotides (Promega, Southampton, UK). Both strands were sequenced. Dye terminator methods (Alta Bioscience, University of Birmingham, Birmingham, UK) were used in the automated sequencing of the cloned fragments using a RoboSeq 4204 machine (Functional Genomics Laboratory, University of Birmingham, Birmingham, UK).

Table 1
Results of PCR with different primers on plasmids classified as IncP-9 by traditional incompatibility testing

Plasmid	Strain[a]	Plasmid size (kb)	Phenotype of plasmid[b]	Hybridization of PCR products[c]			
				*rep*F *rep*R	*ori* 3Fd *rep* 3Rc	*mpfA* 1Fa *korA* 2Ra	*korA* 3Fa *rep* 3Rc
+	*P. putida* BS202	100	degradation of naphthalene to salicylate, silent genes of meta-pathway catechol cleavage	+		+	+
pBS1191	*P. putida* BS3790	100	similar to NPL-1	+	+	+	+
pBS1192	*P. putida* BS3790-E5	60	degradation of salicylate	-	-	-	+
pBS1181	*P. putida* P20	~100	NAH-like, encoding upper and lower pathways of naphthalene and salicylate catabolism	+	+	+	+
pBS240	*P. putida* BS639	160		+	+	+	+
pBS216	*P. putida* BS3710	85		+	+	+	+
pBS265	*P putida* BS394	130	CAP-plasmids encoding degradation of ε-caprolactam	+	+	+	+[d]
pBS267		130		+	+	+	+[d]
pBS268		85		+	+	+	+[d]
p15C	*Pseudomonas* sp. 15C	~100	degradation of naphthalene	+	+	+	+
pMG18	*P. putida*	100	Km^R, Gm^R, Su^R, Cb^R, Hg^R	+	+	(+)	(+)
R2	*P. aeruginosa*	73	Cb^R, Sm^R, Su^R , UV^R	+	+	+	+
pM3	*P. putida*	75	Sm^R, Tc^R	+	+	+	+
pM77	*Paracoccus* sp. 77	75	Sm^R, Tc^R	+	+	+	+
pM80	*Paracoccus* sp. 80	75	Sm^R, Tc^R	+	+	+	+

[a] *P.* = *Pseudomonas*.
[b] Resistance marker abbreviations: Cb^R, carbenicillin; Gm^R, gentamicin; Hg^R, mercuric ion; Km^R, kanamycin; Sm^R, streptomycin; Su^R, sulphonamides; Tc^R, tetracycline; UV^R, UV irradiation.
[c] Hybridization with dioxigenin-labelled, PCR-derived probes (template pMT2). Hybridization signal: +, strong; (+), weak; −, none.
[d] Hybridization of PCR product of the larger size than expected.

3. Results and discussion

3.1. Design of primers specific for IncP-9 plasmids

The sequences of the 8.6-kb mini IncP-9 replicon pMT2 (GenBank accession No. AF078924) [10] derived from 75-kb IncP-9 plasmid pM3 and of pWW0 (GenBank accession No. AJ344068) [29] show overall approximately 85% sequence identity. A series of primers were designed in both orientations in the region of replication, stability and transfer genes, from the *mpfA* (encoding the first gene of a conjugative transfer operon) at one end, to *rep* (replication origin activator) at the other end (Table 2). The regions chosen for amplification covered sequences with the highest homology between pMT2 and pWW0 in regions that were expected to be highly conserved among all IncP-9 plasmids such as the putative *oriV* and genes for the replication protein Rep, partitioning protein ParB or regulatory protein KorA, because of their role in plasmid maintenance. We also chose segments that contain lower similarity and that are likely to be less conserved within the group as a whole. This included intergenic regions between *mpfA* and *parA*, between *korA* and *tolA* (encoding a product similar to the transmembrane protein TolA) or between *tolA* and *res* (putative resolvase gene) that contain multiple binding sites for gene expression or reg-

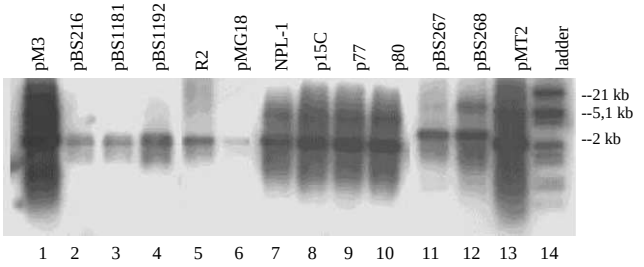

Fig. 1. Southern blot hybridisation of *korA*3Fa–*rep*3Rc PCR products amplified from DNA of different IncP-9 plasmids with pMT2 probe. Lane 14, digoxigenin-labelled ladder.

Table 2
Primer pairs used in PCR experiments

Primer specificity	Name[a]	Sequence (5′→3′)	Product size (bp)	Annealing temp. (°C)	Ref.
16S rDNA	F984GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGG GGCACGGGGGG[b]-AACGCGAAGAACCTTAC	433	53	[27]
	R1378	CGGTGTGTACAAGGCCCGGGAACG			
IncN	*rep* 1	AGTTCACCACCTACTCGCTCCG	164	55	[2]
	rep 2	CAAGTTCTTCTGTTGGGATTCCG			
IncP	*trfA2* 1	CGAAATTCRTRTGGGAGAAGTA	241	57	[2]
	trfA2 2	CGYTTGCAATGCACCAGGTC			
IncQ	*oriV* 1	CTCCCGTACTAACTGTCACG	436	57	[2]
	oriV 2	ATCGACCGAGACAGGCCCTGC			
IncP-9	*rep*F	CCAGCGCGGTACWTGGG	554	54	[4]
	*rep*R	GTCGGCAGCTGCTTGAGCTT			
	mpfA 1Fa	CAGCCAAGTGCGCCAGGTTTG	1085	58	this paper
	parA 1Ra	GCGTTTCATGGTGACCTTCC			
	parB 2Fa	GGAAATGAAACGGGCCTGC	1365	58	this paper
	korA 2Ra	GTCATGGGTCAACGTCCTTGG			
	korA 3Fa	GCAGACCCATTCCATGACCACC	1167	61	this paper
	res 3Ra	CTCTGCCGCTCCCGCCG			
	ori 3Fd	CCACCGACACTGATGGTCTG	800	54	this paper
	rep 3Rc	ACCGTGATGCGTATTCGTG			
	mpfA 1Fa	CAGCCAAGTGCGCCAGGTTTG	3150	58	this paper
	korA 2Ra	GTCATGGGTCAACGTCCTTGG			
	korA 3Fa	GCAGACCCATTCCATGACCACC	2108	53	this paper
	rep 3Rc	CCACCGACACTGATGGTCTG			

[a]F (1), forward primer; R (2), reverse primer.
[b]GC clamp (up to the hyphen).

ulatory proteins, where more sequence drift may be tolerated. However, the primers were generally placed in protein coding regions flanking these proposed variable regions and designed to match perfectly (100%) with the sequences of both pMT2 and pWW0, assuring the specific amplification of the DNA fragments spanned. They all worked efficiently and gave specific products on both pMT2 and pWW0 which were checked by DNA sequencing.

3.2. Specificity of IncP-9 primers and application to different sample types

The specificity of all IncP-9 primers was tested first by PCR on representatives of other incompatibility groups: IncFI, IncFII, IncU, IncX, IncN, IncP-1α, IncP-1β, IncP-2, IncP-7, IncP-13, IncQ, IncW and IncZ. With purified plasmid DNA no PCR products were obtained. This confirmed that the level of sequence similarity between the IncP-9 primers and this range of representative plasmids is insufficient to prime PCR. With total DNA from bacteria carrying these test plasmids non-specific PCR products were occasionally found but in no case did the product hybridise with the respective probe generated by PCR amplification of pMT2. It is therefore clearly important to use hybridisation to check PCR products when using this technique to establish whether a new plasmid belongs to the IncP-9 group or whether a strain contains an IncP-9 plasmid.

To demonstrate the value of the new primers as tools for assessing spread, and characterising diversity, of these particular elements we also attempted to confirm that the primer systems can unequivocally identify the plasmid group for which they were designed. Therefore they were used to amplify products from various IncP-9 plasmids encoding the degradation of naphthalene (NAH) or ε-caprolactam (CAP), isolated from oil-polluted soil in Russia, as well as those carrying resistance properties, found in US and Japanese medical units (pMG18, R2), sewage and soil (pM3) and water reservoirs (plasmids pM77 and pM80) in Belarus. Both plasmid and total cellular DNA from bacteria carrying the appropriate plasmids were used as templates. All combinations of primers tested yielded a PCR product whose identity was confirmed by Southern hybridisation with respective probes derived from pMT2, for the majority of the plasmids (Fig. 1, Table 1). The only exception concerned NAH degradative plasmid pBS1192, where only the *korA*3Fa–*rep*3Rc pair of primers resulted in amplification. Strong hybridisation with pMT2 probes was observed for CAP plasmid DNA amplified with *rep*F–*rep*R and *korA*3Fa–*rep*3Rc despite the larger size of PCR product than was expected (see below). Extra minor bands observed for some plasmids indicate that priming may also occur occasionally at other sites in the region of the intended target.

3.3. Characterisation of IncP-9 replicons by RFLP analysis of PCR products

PCR on DNA of three of the IncP-9 plasmids with

Table 3
Screening of directly extracted DNA originating from different environments by means of PCR with subsequent Southern blot hybridisations for the presence of IncP-9 plasmids

Sample origin		Hybridisation of PCR products with IncP-9-specific probes[a]				
		*rep*F–*rep*R	*ori3*Fd–*rep3*Rc	*mpfA*1Fa–*parA*1Ra	*parB* 2Fa–*korA*2Ra	*korA*3Fa–*res*3Ra
Manure	Broiler chicken	–	–	–	–	–
	Broiler+flavomycin	++	+	–	–	–
	Layer chicken	–	–	–	–	–
	Layers+Zn-bacitracin	–	–	–	–	–
	Pig+neomycin	++	++	–	–	–
	Pig control group	++	++	–	–	+
	Cattle+monensin	+	+	–	–	–
	Cattle control group	–	–	–	–	–
Rhizosphere	Dossenheim+Sm	–	–	–	–	–
	Dossenheim control	–	–	(+)	(+)	–
	Ens+Cu (+manure)	–	–	–	–	–
	Ens control (+manure)	–	–	–	–	–
Coastal water	Fishfarm at Epidauros	–	–	–	–	–
	Wastewater outflow	–	–	–	–	–
	Fleves Island, pristine	–	–	–	–	–
	Eretria, pristine	+	(+)	–	–	–
Soil	Dossenheim+Sm	+	(+)	++	+	–
	Dossenheim	–	–	–	–	–
	Cotswold, pristine	–	–	++	–	–
	Droitwich (+sludge)	–	–	(+)	+	–
Sewage	Wavre, community	–	–	–	–	–
	Erasme, hospital	–	–	–	–	–
	Rosière, community	–	–	–	–	–
	Ghent, hospital	–	–	–	–	–

[a] –, no hybridisation; (+), weak hybridisation; +, hybridisation; ++, strong hybridisation.

primers pairs *korA*3Fa–*rep*3Rc and *rep*F–*rep*R, which cover segments overlapping a part of the *rep* gene, gave products of greater length than that obtained for pMT2 or pWW0 (Table 1). This suggested unanticipated differences in this region between members of the group. To assess these and other differences, RFLP analysis was performed on fragments amplified with primers *korA*3Fa and *rep*3Rc from selected IncP-9 plasmids on the basis of diverse sources and phenotypes carried. Since we expected the *rep* region of the IncP-9 'core region' to be the most highly conserved, we also examined the adjacent sequence flanked by primers *mpfA*1Fa–*korA*2Ra that we expected to exhibit more diversity. The pattern of *Hae*III restriction digest of the respective PCR products (Fig. 2) clearly showed that these plasmids, although related, vary not only within the catabolic/resistance functions they carry, but also in the region responsible for replication and stability. This indicates the value of using two pairs of PCR primers for IncP-9-specific amplification of this core region followed by restriction analysis of the products for grouping IncP-9 isolates that come from different ecological niches. Fig. 2 shows that all tested plasmids fall into three patterns given by each PCR product which correlates with the phenotype encoded, giving us sufficient grounds for future sub-classification. Therefore the restriction analysis of the plasmid backbone amplified with the primers we proposed seems to be an efficient approach to provide the basis for sequencing-selected segments of chos-

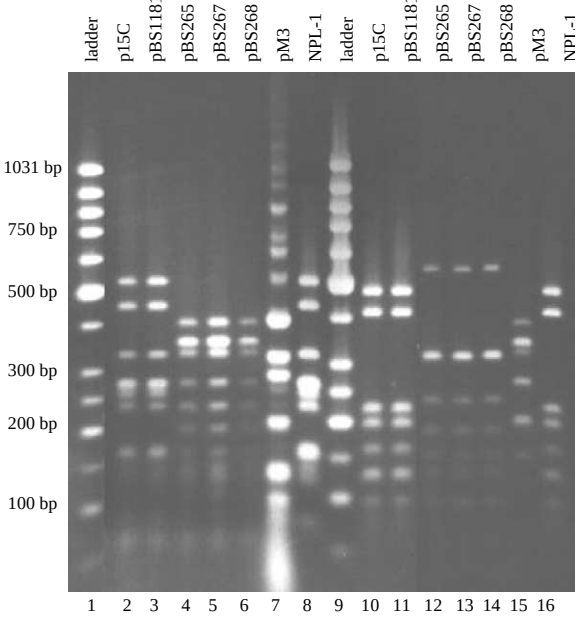

Fig. 2. *Hae*III restriction patterns of PCR-amplified fragments of different IncP-9 plasmids obtained with *mpfA*1Fa–*korA*2Ra (lanes 2–8) and *korA*3Fa–*rep*3Rc (lanes 10–16) primers. Lanes 1 and 9, 50-bp DNA ladder. Note that in some cases there is clearly incomplete digestion (for example for pM3), so some bands correspond to partial digestion products, but these can generally be distinguished easily by their relative intensity.

en plasmids essential for detailed phylogenetic studies of the IncP-9 group. Our studies to date suggest that the integrity of the replication and maintenance region covered by these new PCR primers is necessary for plasmid survival. Therefore, in the absence of recombination between different IncP-9 plasmids the RFLP patterns of their basic replication and maintenance region are likely to be relatively stable during acquisition of additional novel genes in response to changing environmental conditions [30] and therefore may help with plasmid identification, giving a useful view of plasmid relationships. Conversely, these differences detected by the RFLP analysis may also be useful for assessing recombination between different IncP-9 plasmids.

3.4. Application of the IncP-9 primers for detection of replicon-specific regions in total community DNA

The investigation of prevalence of plasmids belonging to the IncP-9 group was carried out for samples originating from a range of environments, in most cases characterised by the presence of strong selective pressure, caused by antibiotics, metals and organic pollutants. To search for IncP-9-like plasmids we used the *rep* primer pair that had already been tested [4] as well as new pairs of oligonucleotides (Table 2), that amplified short segments of genes across the core replication and partitioning region exhibiting over 85% sequence identity between pMT2 and pWW0. The two pairs *mpfA*1Fa–*korA*2Ra and *korA*3Fa–*rep*3Rc that appear to be efficient for analysis of IncP-9 isolates were not considered appropriate for screening of community DNA since the size of fragments they amplify would limit detection to intact DNA which is, however, likely to be sheared during extraction. Five pairs of primers, *rep*F–*rep*R, *ori3*Fd–*rep3*Rc, *mpfA*1Fa–*parA*1Ra, *parB*2Fa–*korA*2Ra and *korA*3Fa–*res*3Ra, were used to amplify total community DNA extracted from samples which had been taken from habitats once or several times during the period of monitoring. The presence of sequences specific for the IncP-9 group was judged initially by the amount of PCR products of the expected size visible after agarose gel electrophoresis and confirmed by hybridisation with DNA probes obtained after PCR amplification of fragments of pMT2 flanked by primers used in the screening experiment. Results of Southern blot hybridisations are summarised in Table 3. It should be noted that the variations in strength of signal may not reflect abundance of the plasmid DNA in these signals, but could simply reflect the quality of the template. Reconstruction experiments indicate that a positive signal can be obtained when approximately 0.01% of the bacteria carry the plasmid sequence.

Based on signals obtained by PCR with primers for the *rep* and *ori–rep* regions, IncP-9-related plasmids appeared mainly in manure, showing some bias towards antibiotic-treated environments. They could also be detected in coastal water and streptomycin-treated soil. Interestingly these results were not obtained when the other IncP-9 primers were used. This may suggest the existence of plasmids with related *rep* sequences but different or less closely related auxiliary maintenance functions. The primers also allowed detection of IncP-9-like sequences in a rhizosphere sample that was free from antibiotics and in a range of soil samples (pairs *mpfA*1Fa–*parA*1Ra, *parB*2Fa–*korA*2Ra). The only agreement between primers tested, with the exception of the *korA*3Fa–*res*3Ra pair, was observed in the case of soil treated with streptomycin. This may suggest the existence of closely related replicons in this specific ecological niche. Streptomycin resistance is not a phenotype conferred by many IncP-9 plasmids (Table 1), although pM3 is known to carry this marker and indistinguishable plasmids have been isolated many times from soil in Eastern Europe (M.T., unpublished). The geographical distribution of positive samples is probably not significant at this stage, simply indicating that IncP-9 plasmids can be found all over Europe, but not universally in all samples.

The primers *korA*3Fa–*res*3Ra span two intergenic regions separated by *tolA*, the least conserved gene in the core cluster. They were designed to allow detection of more distantly related IncP-9 sequences. Using this pair of oligonucleotides we only obtained a positive signal for pig manure. Therefore, this particular niche may host a variety of IncP-9-like plasmids sharing the *oriV–rep* segment and having also similar sequence organisation outside the replication and partitioning regions.

Table 4
Presence of plasmids belonging to different incompatibility groups in sewage samples over the Zn treatment

Sample origin	Sampling	IncP-1α (*trfA2*)	IncP-1β (*trfA2*)	IncP-9 (*rep*)	IncN (*rep*)	IncQ (*oriV*)
Sewage of pigs fed with Zn	Day 0	+	(+)	++	+	(+)
	Day 7	+	+	++	+	+
	Day 21	+	+	++	(+)	−
	Day 34	+	+	+	−	−
Sewage of pigs fed without Zn	Day 0	(+)	(+)	++	(+)	(+)
	Day 7	(+)	(+)	++	(+)	(+)
	Day 21	−	(+)	++	−	−
	Day 34	−	(+)	(+)	−	−

−, no hybridisation; (+), weak hybridisation; +, hybridisation; ++, strong hybridisation.

Plasmid diversity analysis performed with *rep*F–*rep*R PCR of total community DNA that was isolated from sewage of piglets whose diets either had or had not been supplemented with zinc, revealed strong and consistent IncP-9 signals from both sample sets (Table 4). This compares with signals for IncN- and IncQ-like sequences, which were weak and inconsistent, and also with IncP-1α,β-like sequences that seemed not to be favoured by the zinc treatment.

The strength of hybridisation signal given by IncP-9-specific amplification of total community DNA from nearly every sample isolated throughout the period of zinc treatment was far stronger than that obtained from other environments tested. We cloned the PCR products of DNA extracted from sewage of the zinc-treated pigs and sequenced three clones for each product. All analysed sequences from clones covering the seven zinc-treated samples showed 100% identity to the pMT2 (pM3) *rep* gene. To attempt to recover these plasmids we performed serial dilutions on the sewage samples from 28- and 34-day zinc-treated pigs as well as untreated controls and plated on a rich agar medium. The resulting colonies appeared relatively homogeneous so we pooled bacteria from the plates and extracted plasmid DNA. A large plasmid could be seen in the zinc-treated samples but not in the control. However, PCR with this DNA using the IncP-9 primers did not yield a product and the plasmid DNA that was visible on the gel did not hybridise with pMT2 *rep*F–*rep*R PCR probe when Southern blotted. A possible explanation would be that IncP-9-like sequences are hosted by bacteria that are in the non-culturable fraction of the bacterial population present in piglet sewage or that do not grow under the cultivation conditions chosen. It may be possible to overcome this problem in the future by capturing IncP-9 plasmids using biparental exogenous isolation into *Pseudomonas putida*, using selection for any of the markers carried by known IncP-9 plasmids [30,31], or triparental mating [32,33] using the ability to mobilise IncQ plasmids between two marked strains as, for example, described for the cryptic rhizosphere plasmid pIP02 [34].

4. Conclusions

In this paper we report the design of new PCR primer pairs for detection and classification of DNA sequences related to the IncP-9 core of replication and stable inheritance genes. We have demonstrated their specificity and their application to representative plasmids of known IncP-9 sample groups. The pairs *mpfA*1Fa–*korA*2Ra and *korA*3Fa–*rep*3Rc are more suitable for characterising of IncP-9 plasmid isolates and examining diversity within the group. Primers *ori3*Fd–*rep3*Rc, *mpfA*1Fa–*parA*1Ra, *parB*2Fa–*korA*2Ra and *korA*3Fa–*res*3Ra when applied to total community DNA enable rapid detection and preliminary assessment of the relationship between IncP-9-like sequences present in various environmental niches. The preliminary results suggest that there are at least three IncP-9 subgroups, in contrast for example to the two IncP-1 subgroups [35,36], although solid conclusions about diversity will depend on extensive DNA sequence comparisons. Given the wide geographical locations (North America, Japan and Europe) from which IncP-9 plasmids have been isolated in the past and their carriage of both degradation and resistance markers [15], it is perhaps not surprising that we found them in a significant number of the environment samples tested from the UK, Continental Europe and the Mediterranean. However, these plasmids are clearly not ubiquitous, nor does their presence correlate only with highly selected environments, especially where direct comparison was carried out with plasmids of the IncP-1, IncN and IncQ (IncP-4) groups [5]. Since these plasmids are of obvious environmental significance, the primers will be important in cataloguing the diversity and phylogeny of IncP-9 plasmids, a project currently underway.

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