Targeted metagenomics demonstrates the ecological role of IS1071 in bacterial community adaptation to pesticide degradation

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Summary

IS1071, an insertion element that primarily flanks organic xenobiotic degradation genes in cultured isolates, is suggested to play a key role in the formation and distribution of bacterial catabolic pathway gene clusters. However, in environmental settings, the identity of the IS1071 genetic cargo and its correspondence to the local selective conditions remain unknown. To respond, we developed a long-range PCR approach amplifying accessory genes between two IS1071 copies from community DNA followed by amplicon sequencing. We applied this method to pesticide-exposed environments, i.e. linuron-treated agricultural soil and on-farm biopurification systems (BPS) treating complex agricultural wastewater, as to non-treated controls. Amplicons were mainly recovered from the pesticide-exposed environments and the BPS matrix showed a higher size diversity compared to the agricultural soil. Retrieved gene functions mirrored the main selection pressure as (i) a large fraction of the BPS amplicons contained a high variety of genes/gene clusters related to the degradation of organics including herbicides present in the wastewater and (ii) in the agricultural soil, recovered genes were associated with linuron degradation. Our

metagenomic analysis extends observations from cultured isolates and provides evidence that IS1071 is a carrier of catabolic genes in xenobiotica stressed environments and contributes to community level adaptation towards pesticide biodegradation.

Introduction

Horizontal gene transfer (HGT) is a prime mechanism by which bacteria acquire abilities to catabolize recalcitrant organic compounds (Top and Springael, 2003). Mobile genetic elements (MGEs), such as plasmids and transposons, not only control the dispersion of catabolic genes but are also the gateway to an open source evolution of metabolic pathways by (re)combination of suitable catabolic gene modules (Iwasaki and Takagi, 2009).

An MGE that shows a remarkably frequent association with genes encoding organic xenobiotic degradation in bacteria is the insertion sequence IS1071. This element was first described bordering the 3-chlorobenzoate catabolic genes on the *Pseudomonas* sp. (renamed as *Comamonas testosteroni*) BR60 plasmid pBRC60 (Fulthorpe and Wyndham, 1992). Since then, it was reported to border various other organic xenobiotic catabolic – often plasmid-encoded – gene clusters in xenobiotic degrading bacteria (Bers *et al.*, 2011; Martinez *et al.*, 2001; Vedler *et al.*, 2004; Sen *et al.*, 2010; Ledger *et al.*, 2006). With its 3.2 kb in length and 110 bp long inverted repeats, IS1071 is a unique IS element and, unlike other IS elements, is categorized as a class II transposon based on the transposase (*tnpA*) gene sequence (Sota *et al.*, 2008). Often IS1071 flanks the catabolic genes at both sites forming a putative composite transposon that in the case of pBRC60 was shown to effectively transpose as an entity between replicons (Fulthorpe and Wyndham, 1992). Plasmids that often carry such apparent IS1071 catabolic composite transposons are the highly promiscuous plasmids of the IncP-1 group, suggesting that IS1071 mediates the recruitment of the catabolic genes by IncP-1 plasmids upon which the composite transposon can be further distributed (Dennis, 2005; Nojiri *et al.*, 2004; Top and Springael, 2003).

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All these conclusions are based on information from cultured strains often isolated from enrichment cultures with the application of highly selective conditions. Less is known about the occurrence of *IS1071* and especially its cargo genes in environmental settings and their complex communities. Unusually high *IS1071* abundances were recorded in organic xenobiotic exposed natural and engineered ecosystems such as agricultural soils treated with pesticides and on-farm biopurification systems (BPSs) that treat pesticide contaminated wastewater at farms, indicating the implication of *IS1071* in genetic adaptation of microbiota towards organic xenobiotic biodegradation in environmental settings and hence the ecological relevance of *IS1071* (Dunon *et al.*, 2013; Dealtry *et al.*, 2014a, 2014b). However, it is unclear whether *IS1071* elements in natural environments are associated with functional genes and whether those accessory genes can be linked with the local selective forces such as the presence of organic xenobiotics. Such knowledge is needed to fully grasp and acknowledge the ecological role of IS elements like *IS1071* as mediators in community adaptation in the environment.

Metagenomics based on shotgun sequencing of community DNA by next-generation sequencing (NGS) is a powerful tool to explore the functional diversity within microbial communities. However, in case of environments with complex communities, such as soils, retrieving gene functions of interest and determining their genetic context from metagenomic sequence data is a challenge especially when the corresponding bacterial populations compose only a small fraction of the total community. The latter is often the case for xenobiotica-degrading bacteria as the xenobioticum only represents a minor fraction of the available carbon for growth. For instance, several recent studies show no major changes in community profiles in pesticide amended environments compared to non-amended controls unless the compound is added at unrealistic high loading rates (Sniegowski *et al.*, 2011a, 2011b; Bers *et al.*, 2013b; Dealtry *et al.*, 2016). A recent metagenomic analysis by Fang *et al.* (2014) supports this. The study revealed the presence of pesticide degradation genes in freshwater and marine sediments, but no information was recovered about their genetic context. Therefore, novel 'targeted metagenomic' approaches are required to collect sequence information on specific subsets of metagenomes (Suenaga, 2012). In this study, we present a novel culture-independent approach to acquire a better understanding of the gene functions carried by *IS1071* in the environment and the role of *IS1071* in microbial community genetic adaptation, beyond the information based on cultured isolates. The approach uses long-range PCR (LR-PCR) combined with NGS that recovers and identifies the accessory genes of *IS1071* composite transposons in environmental DNA (eDNA). The LR-PCR uses primers that target the *tnpA* gene of

IS1071 with primer sequences that are directed outward of the IS element in order to amplify the intermediate DNA between two *IS1071* elements. The LR-PCR method was applied on agricultural soil treated with the herbicide linuron and the same untreated control soil for comparative purposes. Similarly, the method was applied on material from an on-farm BPS in operation and on artificial pristine BPS material. We hypothesized that DNA recovered by our approach is enriched in pesticide catabolic genes in pesticide-exposed environments compared to the control environments.

Results

Optimization of a LR-PCR to recover accessory genes associated with IS1071 composite transposons from eDNA

To design suitable primers for amplification of *IS1071* composite transposon accessory genes by means of LR-PCR, alignment and clustering of available *IS1071* sequences was performed. Clustering of the sequences showed two main subtypes of *IS1071* (subtype I and subtype II), with *IS1071* subtype 1 representing the main fraction (80 of the 91 sequences; Supporting Information Fig. S1). Primers were designed and degenerated such that they recognized both *IS1071* subtypes and matched all 91 sequences. LR-PCR was optimized using IncP-1 plasmids containing *IS1071* composite transposons with different accessory lengths (from 3.7 to 32.9 kb) as templates (Table 1). Using a 16-min elongation time, expected amplicons between 3.5 and 18 kb were recovered with only minor a-specific amplification. Multiple targets were simultaneously recovered as shown with plasmid pADP-1, carrying two *IS1071* accessory genes regions (Supporting Information Fig. S2), and with a mixture of pUO1 and pADP-1 with a total of three accessory genes regions (data not shown). However, using plasmid pl2, containing several *IS1071* composite transposons, only the smaller 3.7 kb region was recovered and not the larger 24.8 kb segment. This suggests that an elongation time of 16 min recovers amplicons with sizes up to 20 kb (data not shown). Increasing the elongation time up to 33 min improved amplicon length but also resulted in amplification of plasmid backbone regions, as observed by the amplification of a 32.9 kb backbone region when plasmid pEST4011 was the template (data not shown). As a proof of concept, LR-PCR was performed on high molecular weight eDNA isolated from BPS Kortrijk material to which a 10-fold dilution series of *E. coli* containing plasmid pNB8c was added. The identity of the 15.1 kb amplicon of pNB8c, containing the *dcaRQTA1A2BC* gene cluster, was confirmed by Southern blot hybridisation using *dcaQ* as a probe. Amplicons of the expected size (in addition to amplicons originating from endogenous *IS1071* accessory gene

Table 1. Plasmids used in this study.

Plasmid	Host	Size (kb)	Size IS1071 accessory regions (bp) (expected size of LR-PCR amplicon)	Relevant characteristics	Accession number
<i>IncP-1β</i>					
pNB8c	<i>Escherichia coli</i>	60	15 097	<i>dcaA1A2</i>	JF274990
pUO1	<i>Delftia acidovorans</i>	67	5 316	<i>dehH1H2</i>	AB063332
pAKD18	<i>Pseudomonas putida</i>	67	12 714	Hg ^R	JN106169
pl2	<i>Escherichia coli</i>	84	3 746; 24 809;	Hg ^R ; <i>dcaA1A2</i>	JF274989
pADP-1	<i>Escherichia coli</i>	109	9 107; 11 324	Km ^R ; <i>atzA</i>	U66917
<i>IncP-1δ</i>					
pEST4011	<i>Alcaligenes xylosoxidans</i>	77	43 198	<i>tdfA</i>	AY540995
<i>IncP-1ϵ</i>					
pEMT3	<i>Escherichia coli</i>	63	17 917	Km ^R ; <i>tdfA</i>	JX469827

For each of the plasmids their relevant characteristics and expected IS1071 LR-PCR amplicons are given. Abbreviations: Km^R, kanamycin resistance; Hg^R, mercury resistance; *atzA*: Atrazine chlorohydrolase; *dcaA1A2*: Dichloroaniline dioxygenase; *dehH1H2*: Haloacetate dehalogenases; *tdfA*: α -ketoglutaric acid dioxygenase.

clusters) were obtained as long as 10^4 copies of pNB8c per gram of material or more were present (Fig. 1). Diluting the eDNA extract 10-fold improved amplification while high concentrations of pNB8c resulted in the less efficient recovery of endogenous targets.

Size range of IS1071 accessory genes in environmental samples as determined by LR-PCR

The optimized LR-PCR was used to determine the diversity of IS1071 composite transposon accessory genes in two types of environments. The first environment was the matrix of a BPS in operation at a farm near the city

of Kortrijk, Belgium that receives a high load of various pesticides (BPS Kortrijk). Samples analysed were taken in March 2011 before the start of the spraying season, during the spraying season in July and September 2011, and after shutting down its operation in December 2011. As a non-contaminated BPS control, a mixture of materials used in BPS mimicking non-treated BPS material (BPS pristine) was analysed in parallel. In advance to the LR-PCR, the relative abundance of IS1071 was determined in the BPS Kortrijk matrix by means of qPCR targeting *tnpA* and was found to be high throughout the season. Although there was merely a threefold change in abundance between samples taken in the middle of the

Fig. 1. Recovery of IS1071 accessory gene LR-PCR amplification products from BPS Kortrijk matrix samples spiked with different amounts of *Escherichia coli* containing plasmid pNB8c as indicated. The actual pNB8c copy numbers were determined using real-time PCR quantifying the *tnpA* gene. LR-PCR was performed on three 10-fold dilutions (indicated above the lanes) of the corresponding eDNA extract. Positive controls included both genomic DNA extracted from *E. coli* containing pNB8c and purified pNB8c plasmid DNA.

A. Agarose gel electrophoresis of recovered amplicons.

B. Southern blot hybridization with *dcaQ* as probe. As negative control (neg. ctr), a non-template control was included in the LR-PCR. A regular (L) and a digoxigenin labelled (DIGIII) size marker were used to estimate amplicons sizes.

spraying season (July and September) and samples from March and December, this difference was significant (ANOVA; $p < 0.05$; Tukey's test; Supporting Information Fig. S3). The second environment was agricultural soil (soil L) sampled from a previously reported soil microcosm (SM) experiment in which the soil was treated with tap water supplemented (microcosms 'L SM A', 'L SM B' and 'L SM C') or not-supplemented (microcosms 'W SM D', 'W SM E' and 'W SM F') with the phenylurea herbicide linuron (Bers *et al.*, 2013b) for 491 days. In microcosm 'L SM B', feeding with linuron was replaced by only tap water from day 285 till day 491. Samples that were analysed were those taken at the end of the experiment at day 491. Linuron treatment of soil L was previously shown to increase the numbers of *IS1071* gene copies substantially (Dunon *et al.*, 2013).

For the LR-PCR method, the size range of recovered amplicons was used as a first measure of diversity. In the BPS Kortrijk samples, the amplicon sizes ranged between 5 and 20 kb. Profiles depended on the sampling position and time of sampling which is explained by the large

matrix heterogeneity in such systems. On the other hand, many common bands can be recognized between samples (Fig. 2A). In contrast, amplicons originating from eDNA from BPS pristine were around 10 kb in length, with a less diverse size range compared to BPS Kortrijk (Fig. 2B). For the agricultural soil, both replicate microcosms treated with linuron till day 491 showed similar LR-PCR amplicons with sizes between 7 and 20 kb. Interestingly, no amplicons were recovered from SM replicates that did neither receive linuron nor from the L SM B replicate for which linuron treatment was ceased from day 285 (Fig. 2C).

NGS analysis of metagenomic LR-PCR amplicons: general observations

LR-PCR amplicons from selected samples were analysed by NGS as a proof of concept. The selected samples included a BPS sample from the September sampling campaign (compartment 4) amid the pesticide spraying season showing a high abundance of *IS1071* copies, soil from linuron microcosm L SM A

Fig. 2. Agarose gel electrophoresis visualization of *IS1071* accessory gene LR-PCR amplicons recovered from BPS Kortrijk, BPS pristine and the linuron treated soil microcosm experiment.

A. Samples from BPS Kortrijk were taken in the course of the agricultural season of 2011 in March, July, September and December. At each time point, high molecular weight eDNA was isolated from four samples taken at different locations (compartments 1–4) of the BPS and used as a template for LR-PCR.

B. High molecular weight eDNA was isolated from a freshly made pristine BPS setup and used as a template for the LR-PCR.

C. High molecular weight eDNA was isolated at day 491 of incubation from all microcosm setups and used as a template for the LR-PCR. Different treatments with and without linuron were performed in triplicate: (i) discontinuous irrigation with sterile tap water (W SM D, W SM E and W SM F) and (ii) discontinuous irrigation with sterile tap water containing 60 mg l⁻¹ linuron (L SM A and L SM C). In microcosm L SM B, after 285 days, linuron supply was ceased and instead sterile tap water was added. Plasmid DNA of pNB8c and pEMT3 carrying *IS1071* composite transposons were included as positive controls with expected amplicon sizes of respectively 15.1 and 17.9 kb. A non-template control was included as negative control (neg. ctr). To estimate amplicon sizes a size marker (L) was included.

representative for the 491 days of continuous linuron application and the BPS pristine sample. Results from the assembly into contigs of HiSeq 2000 reads using Meta-Velvet and Velvet were highly similar for the same k-mer value. Since the k-mer value applicable in Meta-Velvet was limited (≤ 63) at the time of assembly, final assemblies were done with Velvet with k-mer value of respectively 65, 73 and 87. Results are shown in Table 2. A number of contigs, size ranges of contigs and total unique sequence content varied between the samples, with BPS Kortrijk yielding the most contigs (316) and the highest unique sequence content (662 kb). Overall, sizes of contigs ranged between 0.2 and 18.2 kb. The lowest amount of sequence information was recovered from BPS pristine. IS1071 sequences were identified at one or both contig boundaries in 30%, 20% and 50% of the contigs of BPS Kortrijk, L SMA and BPS pristine, respectively and several contigs (with sizes between 1.0 and 17.2 kb) displayed IS1071 IR sequences at both ends (Table 2). Based on the IR sequence, the majority of IS1071 sequences were allocated to subtype 1 IS1071 elements (Table 2). Two contigs (contig 75 and 164) from BPS Kortrijk flanked at both ends by IS1071 IRs, carried as well a subtype 1 as a subtype 2 IS1071 element. The 18 kb contig 255 of BPS Kortrijk was flanked by an IS1071 element with a partial *tnpA* gene with only 87% nucleotide (nt) identity to the representative IS1071 subtype 1 of the haloacetate catabolic plasmid pUO1 (Sota *et al.*, 2003), although the deduced partial transposase shares 94% amino acid (aa) identity with TnpA of pUO1. Similarities with the IS1071 subtype 2 of the

4-chloronitrobenzene catabolic pCNB-1 (Ma *et al.*, 2007) were only 74% and 77% nt and aa identity, respectively.

Identification of accessory genes on metagenomic IS1071 LR-PCR amplicons

To obtain a first overview of the functions encoded by the contigs, ORFs were predicted by RAST (total numbers are shown in Table 2), KEGG Orthology (KO) identifiers were assigned to the deduced protein sequences to classify the proteins into functional categories (Fig. 3). A KO number was assigned to 26%, 25% and 27% of the predicted proteins recovered from BPS Kortrijk, L SM A and BPS Pristine, respectively (Table 2). However, many of the ORFs were assigned a KO that was not linked to a specific functional category, i.e. 23% (44/193), 25% (7/39) and 27% (5/12) of the assigned KO of respectively BPS Kortrijk, L SM A and BPS pristine. Predominant functional categories present in the BPS Kortrijk contigs included xenobiotics biodegradation and metabolism (46 ORFs), amino acid metabolism (40 ORFs) and carbohydrate metabolism (23 ORFs). Predominant categories in the contigs from L SM A were translation (12 ORFs) and xenobiotics biodegradation and metabolism (4 ORFs). Most predicted protein functions found in the BPS pristine contigs were amino acid metabolism (3 ORFs), xenobiotics biodegradation and metabolism (3 ORFs) and carbohydrate metabolism (3 ORFs). KOs from BPS Kortrijk and BPS pristine were classified in more than one category. Especially KOs classified in the amino acid metabolism were also classified in the xenobiotics metabolism and carbohydrate metabolism. For instance, for BPS Kortrijk, seven ORFs were classified in the amino acid, carbohydrate and xenobiotic metabolism. Those seven ORFs were annotated as either an acetyl-CoA C-acetyltransferase or an aldehyde dehydrogenase, involved in more downstream reactions of catabolic pathways. In contrast, ORFs that were solely categorized to xenobiotic metabolism included enzymes catalysing upstream reactions in xenobiotic degradation pathways, e.g. aromatic ring-hydroxylating dioxygenases and monooxygenases. Besides metabolic functions, BPS Kortrijk showed proteins associated with membrane transport and cellular community (Fig. 3) of which 15 ORFs were annotated in both categories being related to ABC-transporter systems and quorum sensing. Moreover, two ORFs were categorized in the antimicrobial resistance category.

A more in-depth analysis of the predicted protein sequences was done by examining the presence of Hidden Markov Models (HMM) using hmmscan and protein similarities using BLASTp against the UniProtKB/Swiss-Prot database and non-redundant protein database (Tables 2 and 3 and the Supplementary Annotation File). Proteins were recognized as such on 215, 50 and

Table 2. Summary of the analysis of the NGS data obtained from the LR-PCR amplicons recovered from the three studied ecosystems.

	BPS Kortrijk	L SM A	BPS pristine
Clean sequencing data (Mbp)	873	503	535
Assembled sequence data (kb)	662	131	44
Contigs	316	73	17
Size range contigs (kb)	0.2–18.2	0.2–14.0	0.2–10.7
Contigs with IS1071 IR (contigs with 2 IRs)	105 (18)	16 (2)	9 (3)
% IS1071 subtype 1	89.1	94.4	100
ORFs	742	158	44
ORFs with KO	193	39	12
ORFs with known function BLASTp [nr]	393	88	28
ORFs with HMM	517	94	26

Reported is the amount of data used after quality filtering of the sequencing reads, the total amount of sequence data after assembly, the number of contigs, size range of the contigs, number of contigs with IS1071 IRs and the occurrence of IS1071 subtype 1. Also, the number of predicted open reading frames (ORFs) by RAST is given. Annotations of the ORFs were made by assigning either a KO number using blastKOALA, similar known protein function using BLASTp or the retrieval of Hidden Markov Models (HMM) using hmmscan.

Fig. 3. KEGG orthology (KO) classification of predicted proteins identified on the sequenced IS1071 LR-PCR amplicons of BPS Kortrijk, L SM A and BPS pristine. BlastKOALA (KEGG Orthology and Links Annotation) was used to assign KOs to the predicted proteins based on protein similarities using the reconstruct pathway output. Numbers represent the number of protein sequences that were assigned to that specific KO category. Total numbers of proteins assigned with a KO annotation were 262 (35.3%) for BPS Kortrijk, 51 (32.3%) for L SM A and 15 (34.1%) for BPS pristine.

11 contigs in BPS Kortrijk, L SM A and BPS Pristine respectively. In total, 41%, 26%, and 45% of the contigs originating from BPS Kortrijk, L SM A and Pristine BPS, respectively contained at least one ORF that encodes a putative function showing substantial aa similarity to catabolic enzymes (Supplementary Annotation File). The catabolic functions included more upstream functions of catabolic pathways as well as more downstream functions. Upstream functions included aromatic ring-

hydroxylating Rieske dioxygenases or monooxygenases (17 ORFs in BPS Kortrijk, 3 ORFs in L MS A and 0 ORFs in BPS Pristine), taurine/ α -ketoglutarate dioxygenases (6 in BPS Kortrijk and 0 in L MS A), enzymes that act on N-containing functional groups like amidohydrolases/amidases (10 ORFs in BPS Kortrijk, 2 ORFs in L SM A), nitrile hydratases (3 ORFs in BPS Kortrijk) and nitrilases (1 ORF in BPS Kortrijk) and enzymes involved in chlorine removal (2 contigs in BPS Kortrijk). Several of these

could be directly (with more than 99% aa identity) linked with known enzymes involved in pesticide degradation like HylA (contig 290 in BPS Kortrijk and contig 22 in L SM A) catalysing linuron hydrolysis (Bers *et al.*, 2013a) and AtzA (contig 260 in BPS Kortrijk) catalysing atrazine chlorine hydrolysis (de Souza *et al.*, 1998). Others showed moderate similarity to known pesticide biodegradation enzymes like TfdA/RdpA (contigs 132, 205, 265, 298 and 302 in BPS Kortrijk) and TfdB (contig 231 in BPS Kortrijk) involved in phenoxy herbicide biodegradation (Fukumori and Hausinger, 1993; Ledger *et al.*, 2006; Müller *et al.*, 2006). Downstream pathway enzymes included aromatic ring cleavage enzymes and enzymes that are involved in the further conversion of the (substituted) aromatic cleavage products to Krebs cycle intermediates. Similar to the upstream catabolic functions, several of these proteins could be directly linked to pesticide biodegradation like proteins encoding chloroaniline dioxygenase (contig 250 in BPS Kortrijk and contig 23 in L SM A) involved in the degradation of several pesticide groups including phenylurea herbicides such as linuron (Bers *et al.*, 2013a) and enzymes of the modified *ortho*-cleavage pathway for chlorocatechol degradation (contigs 70, 117, 293 and 309 in BPS Kortrijk and contigs 21 and 33 in L SM A) for instance involved in degradation of chlorinated aromatic pesticides (Kumar *et al.*, 2016). Other downstream pathway enzymes were enzymes that could be linked to lipid (like cholesterol) and fatty acid metabolism (e.g. aldehyde dehydrogenase and acetyl-CoA transferase), a downstream pathway that is also employed in pesticide biodegradation.

Several contigs showed ORFs that together composed a recognizable catabolic holoenzyme or even a complete or partial catabolic pathway. Examples in the BPS Kortrijk sample are contigs 5, 44, 117, 250, 293 and 318. Contig 5 contained two ORFs that encode the α and β -subunit of a non-corrinoid cobalt nitrile hydratase holoenzyme forming a functional nitrile hydratase as well as an ORF that encodes a putative cobalt transporter, which might be involved in the acquisition of cobalt for the nitrile hydratase (Kobayashi and Shimizu, 1998). Contig 44 contains next to an ORF showing homology to nitrilases, a cluster of genes that together encodes all components of a two-subunit urease as well as accessory proteins that participate in the regulation of nickel delivery and maturation of the urease (Lee *et al.*, 1992). Contig 117 contains all the ORFs required for a functional chlorocatechol *ortho*-cleavage pathway (Bers *et al.*, 2013a), contig 250 for a functional (chloro)-aniline dioxygenase multicomponent enzyme (Król *et al.*, 2012), contig 293 for a pathway degrading a sulfonated aromatic (Halak *et al.*, 2007) and contig 318 for a 4-chlorobenzoate-CoA ligase dehalogenating 4-chlorobenzoate (Layton *et al.*, 1992). In L SM A, contig 21, carries, like contig 117 in BPS Kortrijk, all ORFs

required for a functional chlorocatechol *ortho*-cleavage pathway while contig 23, like contig 250 in BPS Kortrijk shows parts of a (chloro)-aniline dioxygenase holoenzyme. In the BPS pristine sample, no catabolic functions could be directly assigned to any of the putative catabolic ORFs.

Non-catabolic proteins were recognized on most of the contigs, including those containing catabolic ORFs. A majority of these ORFs showed homology at the aa level with regulatory proteins or transport proteins and in many cases homology was found with regulatory (like LysR, IclR and MarR family proteins) and transport proteins involved in aromatic catabolism indicating that they might be involved in substrate uptake for and regulation of the neighbouring catabolic genes. A particular gene function that was identified in a large number of contigs in all three samples was a gene function whose translation product showed identity with extra-cytoplasmic solute receptors belonging to the Bug family. Similar ORFs with unknown function were previously identified in gene clusters encoding the modified chlorocatechol *ortho*-cleavage pathway, a central pathway in chloroaromatic biodegradation (van der Meer *et al.*, 1991; Shin and Spain, 2009). Other non-catabolic functions were related to MGEs. A vast amount of ORFs coded for putative IS element transposase functions, most of which belonged to unknown families, but some belonged to the IS21, IS4 and IS66 families. Other MGE related functions were putative plasmid backbone functions. Most of these plasmid backbone gene functions are gene functions such as KlcA, KlcB, KorC, KleA, KleE and KleF encoded by the replication module of IncP-1 plasmids. Highest identity was found with those of IncP-1 plasmids of the IncP- β 1 and IncP-1 ϵ groups (Schlüter *et al.*, 2003). Other contigs showed plasmid backbone functions that were not related to IncP-1 plasmids. For instance, two ORFs in the BPS Kortrijk contig 144 showed high aa identity with the plasmid mobilization proteins MobL and MobS of the IncQ related *Thiobacillus ferrooxidans* plasmid pTF1 (Drolet *et al.*, 1990). Interestingly, with a few exceptions, contigs carrying transposase functions and especially plasmid functions did not contain ORFs with other functions (Supporting Information Table S1). Other ORFs that could be linked to non-catabolic functions were genes showing similarity at the aa level with multidrug efflux transporters (contig 1 in BPS Kortrijk, 64%), proteins involved in mismatch repair (contig 25 in BPS Kortrijk, MutS, 99%), transglycosylase protein (contig 8 in BPS Kortrijk, 39%; contig 39 in L MS A, 33%) and lysis protein (contig 37 in L MS A, muramidase, 38%), ribosomal-proteins (r-proteins; contigs 43 and 44 in L MS A, 48–71%), UV protection protein (contig 26 in BPS Kortrijk, lpmB, 38%), mutagenesis proteins (contig 26 in BPS Kortrijk, UmuC, 38%), uncharacterized putative membrane proteins (contig 27 in BPS Kortrijk, 51%;

Table 3. Overview of ORFs with predicted functions in degradation of organic xenobiotics.

ORF	Contig	Name	Position	#AA	Predicted function	hmmscan		BLASTp		AA ID (%)	Accession n°
						Description		Description (Identifier)	E-value		
18	<i>BPS Kortrijk</i> contig005	<i>nthA</i>	1 382–2 095	237	Nitrile hydratase, α -subunit	Nitrile hydratase, alpha chain		Cobalt-containing nitrile hydratase subunit α [<i>Comamonas testosteroni</i>]	9.56E-170	98.3	KGH21291
19	contig005	<i>nthB</i>	2 106–2 765	219	Nitrile hydratase β -subunit	Nitrile hydratase beta subunit		Nitrile hydratase [<i>Comamonas testosteroni</i>]	8.71E-134	98.6	WP_034398444
20	contig005		2 762–3202	146	Nitrile hydratase β -subunit	Nitrile hydratase beta subunit		Conserved hypothetical protein [<i>Bordetella petrii</i>]	8.29E-101	100	CAP41749
26	contig007		1 385–936	149	Dioxygenase β -subunit	SnoL-like domain		Ring-hydroxylating dioxygenase β -subunit [uncultured bacterium]	7.19E-101	98.7	WP_031942745
27	contig007		2 605–1 400	401	Dioxygenase α -subunit	Rieske [2Fe–2S] domain		Ring-hydroxylating dioxygenase α -subunit [uncultured bacterium]	0	100	WP_031942746
70	contig026		4 429–3 170	419	Thiolase	Thiolase, N-terminal domain		Thiolase [<i>Rhodospseudomonas palustris</i>]	1.83E-175	62.4	WP_011500473
99	contig032		45–410	121	Flavodoxin reductase	2Fe–2S iron–sulfur cluster binding domain		Flavodoxin reductase family protein [<i>Variovorax</i> sp. CF313]	3.06E-61	78.5	WP_007836382
114	contig039		3 690–4 127	145	Glyoxalase	Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily Carbon-nitrogen hydrolase		Glyoxalase [<i>Alcaligenes</i>]	1.97E-75	76.6	WP_022983640
123	contig044		757–1 638	293	Nitrilase/aliphatic amidase			Hydratase [<i>Burkholderia multivorans</i>]	0	100	WP_006402485
128	contig044	<i>ureAB</i>	6 236–6 929	571	Urease subunit $\beta\gamma$	Urease beta subunit		Urease subunit $\beta\gamma$ [<i>Burkholderia multivorans</i>]	1.02E-160	100	WP_006402480
129	contig044	<i>ureC</i>	6 929–8 644	571	Urease subunit α	Amidohydrolase family 1		Urease subunit α [<i>Burkholderia multivorans</i>]	0	100	WP_006402479
138	contig046		45–410	121	Flavodoxin reductase	2Fe–2S iron–sulfur cluster binding domain		Flavodoxin reductase family protein [<i>Variovorax</i> sp. CF313]	3.60E-58	75.2	WP_007836382
172	contig070		111–908	265	Maleyl acetate reductase	Iron-containing alcohol dehydrogenase		Maleylacetate reductase [<i>Ottowia thiooxydans</i>]	6.13E-140	76.6	WP_028604005
202	contig086	<i>tsaB2</i>	18–332	104	Flavodoxin reductase	2Fe–2S iron–sulfur cluster binding domain		Toluene-4-sulfonate monooxygenase system reductase subunit TsaB2 [<i>Comamonas testosteroni</i>]	1.96E-66	99.0	Q9AHG2
212	contig100	<i>nhal</i>	1 947–829	372	Homogentisate 1,2-dioxygenase	Homogentisate 1,2-dioxygenase		Dioxygenase [<i>Methylobaculum zealandicum</i>]	2.77E-140	53.8	WP_026610119
214	contig100		4 531–3 218	437	Amidohydrolase	Amidohydrolase family 2		Amidohydrolase [<i>Burkholderia</i>]	0	83.6	WP_028198123
244	contig115		2 662–1 214	482	Aldehyde dehydrogenase	Aldehyde dehydrogenase		Aldehyde dehydrogenase [<i>Bordetella petrii</i>]	2.47E-169	54.1	WP_026639683
252	contig117	<i>ccdE</i>	721–149	190	Dienelactone hydrolase	Dienelactone hydrolase family		Dienelactone hydrolase [<i>Variovorax</i> sp. WDL1]	1.67E-136	100	AGF25467
253	contig117	<i>ccdD</i>	2 057–840	405	Chloromuconate cycloisomerase	Enolase C-terminal domain-like		Chloromuconate cycloisomerase [<i>Variovorax</i> sp. WDL1]	0	100	AGF25468

Table 3. Continued

ORF	Contig	Name	Position	#AA	Predicted function	hmmscan		BLASTp		E-value	AA ID (%)	Accession n°
						Description	Description (identifier)	Description (identifier)	Description (identifier)			
254	contig117	ccdF	2 670–2 020	216	Maleylacetate reductase	Iron-containing alcohol dehydrogenase		Maleyl acetate reductase [Variovorax sp. WDL1]		5.05E-138	99.5	AGF25469
260	contig117	ccdC	6 142–5 396	248	Catechol 1,2-dioxygenase	Dioxygenase		Chlorocatechol 1,2-dioxygenase [Variovorax sp. WDL1]		0	100	AGF25473
265	contig121		1 311–493	272	Protocatechuate 4,5-dioxygenase subunit beta	Catalytic LigB subunit of aromatic ring-opening dioxygenase		Protocatechuate 4,5-dioxygenase subunit β [Hydrocarboniphaga effusa]		4.00E-91	51.8	WP_007184425
272	contig122	tsaC1	32–658	208	Toluenesulfonate zinc-independent alcohol dehydrogenase	Enoyl-(Acyl carrier protein) reductase		4-formylbenzenesulfonate dehydrogenase TsaC1 [Comamonas testosteroni]		6.30E-151	100	P94681
281	contig132		123–371	82	Dioxygenase	Taurine catabolism dioxygenase Taud, TtdA family		Taurine catabolism dioxygenase Taud/TtdA [Thiothrix nivea]		1.70E-9	46.3	WP_002710665
308	contig151		289–38	83	Dioxygenase			Dioxygenase [Cupriavidus basilensis]		2.61E-26	63.4	WP_017224577
332	contig168	tsaM2	41–346	101	Toluene-4-sulfonate monoxygenase system			Toluene-4-sulfonate monoxygenase system iron-sulfur subunit TsaM2 [Comamonas testosteroni]		2.47E-64	99.0	Q9AHG3
334	contig171		67–735	222	Terephthalate dihydrodiol dehydrogenase	Pyridoxal phosphate biosynthetic protein PdxA		Terephthalate dihydrodiol dehydrogenase [Comamonas testosteroni CNB-2]		2.00E-110	75.0	Q3C1E1
335	contig171		732–1 748	338	Terephthalate 1,2-dioxygenase reductase	Oxidoreductase NAD-binding domain		Terephthalate 1,2-dioxygenase reductase component [Variovorax sp. OK605]		2.00E-161	67.0	WP_027017065
339	contig171	ISF6_0224	3 295–5 073	592	Mono(2-hydroxyethyl) terephthalate hydrolase	Tannase and feruloyl esterase		Mono(2-hydroxyethyl) terephthalate hydrolase [Ideonella sakaiensis 201-F6]		0	76.0	A0A0K8P8E7.1
348	contig176		679–1 956	425	Dioxygenase α -subunit			Hypothetical protein [Ottowia thiooxydans]		0	95.5	WP_028605020
351	contig176	vanB	3 438–4 562	374	Vanillate O-demethylase oxidoreductase	2Fe-2S iron-sulfur cluster binding domain		Vanillate O-demethylase oxidoreductase [Ottowia thiooxydans]		0	98.8	YP_009063612
360	contig180		1 936–1 007	309		2Fe-2S iron-sulfur cluster binding domain		Ferredoxin [Ottowia thiooxydans]		0	89.6	WP_028604719
364	contig181		2 273–1 647	208	Glyoxalase	Glyoxalase/Bleomycin resistance protein/		Glyoxalase [Burkholderia sp. USM B20]		2.80E-112	80.5	WP_044845166
376	contig182		11 089–10 466	207	Glyoxalase	Dioxygenase superfamily Glyoxalase/Bleomycin resistance protein/		Putative dioxygenase [Burkholderia phenoliruptrix BR3459a]		1.31E-111	79.5	AFT86047
406	contig196		1 753–665	362	Dioxygenase α -subunit	Dioxygenase superfamily Rieske [2Fe-2S] domain		Hypothetical protein [Ottowia thiooxydans]		0	93.1	WP_028604720
434	contig205		1 294–2 244	316	Dioxygenase	Taurine catabolism dioxygenase Taud, TtdA family		Hypothetical protein [beta proteobacterium]		1.07E-129	62.2	WP_028820417
436	contig205		2975–3751	258	HMGL-like	4-hydroxy-2-oxovalerate aldolase		4-hydroxy-2-oxovalerate aldolase [Mumia flava]		6.88E-159	93.1	WP_039353545

(Continues)

Table 3. Continued

ORF	Contig	Name	Position	#AA	Predicted function	hmmscan		BLASTp		AA ID (%)	Accession n°
						Description	Description	Description (Identifier)	E-value		
465	contig215		5 074–4 124	316	Dioxygenase β -subunit	2Fe–2S iron–sulfur cluster binding domain		Hypothetical protein [Xenophilus azovorans]	9.91E-111	57.1	WP_038212112
466	contig215		6 329–5 079	416	Dioxygenase α -subunit	Rieske [2Fe–2S] domain		Hypothetical protein [Xenophilus azovorans]	0	70.1	WP_038212110
473	contig215		12 546–11 716	276	Dioxygenase	Taurine catabolism dioxygenase Taud, TfidA family		Hypothetical protein [Xenophilus azovorans]	1.53E-93	55.0	WP_038211985
494	contig227		1598	3	Cyclohexanone monooxygenase	Pyridine nucleotide-disulphide oxidoreductase		Cyclohexanone monooxygenase [Hydrogenophaga intermedia]	0	81.4	WP_035622880
502	contig230		1 173–2 225	350	Vanillate monooxygenase	Rieske [2Fe–2S] domain		Vanillate monooxygenase [Sphingomonas sp. YL-JM2C]	7.57E-91	47.2	WP_029995346
510	contig231		4 059–2 227	610	Phenol monooxygenase	FAD binding domain		Hypothetical protein [Burkholderia sp. WSM4176]	0	57.1	WP_035471484
529	contig247		1 109–2 014	301	Protocatechuate 3,4-dioxygenase β -subunit	Dioxygenase		6-chlorohydroxyquinol-1,2-dioxygenase [Acidovorax sp. CF316]	3.81E-170	82.6	WP_007855039
532	contig247		2 658–3 734	358	Alcohol dehydrogenase	Iron-containing alcohol dehydrogenase		Maleylacetate reductase [Variovorax paradoxus]	0	84.9	WP_018904323
541	contig250	tadQ	30–749	254	Aniline dioxygenase (glutamine synthetase unit)	Glutamine synthetase, catalytic domain		Glutamine synthetase [Delftia tsuruhatensis]	0	99.2	AAX47239
543	contig250	tadT	913–1674	253	Aniline dioxygenase (amino group transferase unit)	Glutamine amidotransferase class-I		Amino group transfer protein [Delftia tsuruhatensis]	7.00E-89	89	AAX47240.1
544	contig250	dcaA1	1 711–3 060	449	Dichloroaniline dioxygenase large subunit	Rieske [2Fe–2S] domain		Dichloroaniline dioxygenase large subunit [Variovorax sp. SRS16]	0	91	AE020097.1
545	contig250	dcaA2	3 126–3 701	191	Dichloroaniline dioxygenase small subunit	Ring-hydroxylating β -subunit		Aniline dioxygenase small subunit [Variovorax sp. WDL1]	1.31E-119	99.5	AGF25495
560	contig255	nagI	10 226–9 252	324	Gentisate 1,2-dioxygenase	Cupin domain		Gentisate 1,2-dioxygenase [Cupriavidus basilensis]	2.69E-135	67.6	AUG22293
572	contig260	atzA	1 443–2 867	474	Atrazine chlorohydrolase	Amidohydrolase family 1		Atrazine chlorohydrolase [ij-proteobacterium CDB21]	0	99.8	BAD69555
579	contig264		23–736	237	2-nitropropane dioxygenase	Nitronate monooxygenase		2-nitropropane dioxygenase [Comamonas testosteroni]	8.78E-165	98.7	WP_003060874
582	contig265		1 996–716	426	Dioxygenase α -subunit	Rieske [2Fe–2S] domain		Hypothetical protein [Xenophilus azovorans]	0	62.9	WP_038215128
585	contig265		3 143–4 105	320	Dioxygenase	Taurine catabolism dioxygenase Taud, TfidA family		hypothetical protein [ij-proteobacterium]	5.41E-116	61.3	WP_028820417
590	contig266		857–2 122	421	Dioxygenase α -subunit	Rieske [2Fe–2S] domain		Hypothetical protein [Xenophilus azovorans]	0	70.7	WP_038215128
615	contig273		608–2 095	495	Amidase	Amidase		Aryl acylamidase [bacterium CSBL00001]	0	100	ACP39716
629	contig276		3 302–2 055	415	Dioxygenase α -subunit	Rieske [2Fe–2S] domain		Hypothetical protein [Ottowia thiooxydans]	0	100	WP_036596184
645	contig290	hylA	127–1 881	584	Linuron hydrolase	Amidohydrolase family 3		Linuron hydrolase [Variovorax sp. PBS-H4]	0	100	AGF25501

Table 3. Continued

ORF	Contig	Name	Position	#AA	Predicted function	hmmscan	BLASTp		E-value	AA ID (%)	Accession n°
						Description	Description (identifier)				
648	contig290	<i>phyH</i>	2 704–1 838	288	Phytanoyl-CoA dioxygenase	Phytanoyl-CoA dioxygenase (PhyH)	Phytanoyl-CoA dioxygenase [Variovorax sp, WDL1]	0	100	AGF25453	
654	contig293	<i>pcaH2</i>	30–659	209	Protocatechuate dioxygenase, β -subunit	Dioxygenase	Protocatechuate dioxygenase-like beta subunit [Hydrogenophaga sp, PBC]	3.24E-130	85.8	CBZ13177	
655	contig293		748–2 004	418	3-carboxy-cis,cis-muconate cycloisomerase	Lyase	Hypothetical protein [Ottowia thiooxydans]	4.93E-110	51.5	WP_028604991	
656	contig293		2 042–2 929	295	2-pyrone-4,6-dicarboxylic acid hydrolase	Amidohydrolase	Hypothetical protein [Ottowia thiooxydans]	9.09E-133	66.9	WP_036595361	
657	contig293		2 926–3 990	354	Alcohol dehydrogenase	Iron-containing alcohol dehydrogenase	Maleylacetate reductase [Pseudomonas resinovorans]	2.00E-42	59	WP_016493058	
659	contig294		841–203	212	Probable phenylacetic acid degradation NADH oxidoreductase PaaE	2Fe–2S iron–sulfur cluster binding domain	Electron transfer protein [Delftia tsuruhatensis]	2.70E-103	100.0	AAX47243	
660	contig296		2 010–493	505	Carotenoid cleavage dioxygenase	Retinal pigment epithelial membrane protein	Carotenoid oxygenase [Comamonas testosteroni]	0	87.9	WP_003052141	
662	contig298		1 811–933	292	2,4-dichlorophenoxyacetate dioxygenase	Taurine catabolism dioxygenase Taud, Tida family	2,4-dichlorophenoxyacetate dioxygenase [Hydrogenophaga sp, PBC]	1.13E-157	79.5	WP_009515748	
669	contig302		970–155	292	Dioxygenase	Taurine catabolism dioxygenase Taud, Tida family	Hypothetical protein [Burkholderia xenovorans]	3.35E-59	46.4	WP_011491428	
687	Contig308		11 140–10 604	178		Amidohydrolase family 3	Amidohydrolase [Xenophilus azovorans]	9,77E-53	52.1	WP_038200272	
693	contig309		486–1 718	271	Dioxygenase α -subunit	Rieske [2Fe–2S] domain	Dioxygenase [Pusillimonas sp, T7-7]	4,79E-129	48.1	WP_013743534	
694	contig309		1711–2184	157	Dioxygenase β -subunit	Snoal-like domain	Hypothetical protein [Bradyrhizobium sp, A11a-2]	3,44E-21	38.8	WP_027583284	
695	contig309		2 196–2 525	410	Toluene-4-monooxygenase subunit TmoC	Rieske [2Fe–2S] domain	2Fe–2S ferredoxin [Ideonella sp, B508-1]	4,83E-27	52.6	WP_022981828	
696	contig309				Toluene-4-monooxygenase, subunit TmoF	Oxidoreductase NAD-binding domain	Oxidoreductase [Alcanivorax pacificus]	1,17E-42	42.7	WP_008734254	
704	contig309		8 964–7 903	353	Alcohol dehydrogenase	Iron-containing alcohol dehydrogenase	Maleylacetate reductase [Herbaspirillum huttiense]	0	76.7	WP_039787278	
705	contig309	<i>ccdC</i>	9 179–8 961	109	Catechol dioxygenase	Dioxygenase	Hypothetical protein P350_16190, partial [Burkholderia cepacia JBK9]	2,16E-32	80.6	KDV23233	
714	contig313		5 348–6 568	72		Amidohydrolase family 2	Hypothetical protein, partial [Solimonas soli]	0	72.6	WP_043114364	
722	contig314		792–1 580	406	2-Pyrone-4,6-dicarboxylic acid hydrolase	Amidohydrolase family 2	Hypothetical protein [Ottowia thiooxydans]	5,27E-149	80.4	WP_036595361	
730	contig316	<i>fcxB</i>	887–1 696	262	4-Chlorobenzoyl CoA dehalogenase	Enoyl-CoA hydratase/isomerase family	4-chlorobenzoyl CoA dehalogenase [Pseudomonas sp, DJ-12]	1,28E-176	95.5	AAC05576	
731	contig316	<i>fcxB</i>	1 709–3 295	269	4-Chlorobenzoyl CoA ligase	AMP-binding enzyme	4-chlorobenzoyl CoA ligase [Alcaligenes sp, AL3007]	0	82.5	YP_009079291	
735	contig316	<i>fcxB</i>	5 693–6 121	528	4-Hydroxybenzoyl CoA thioesterase	Thioesterase-like superfamily	4-hydroxybenzoyl CoA thioesterase [Pseudomonas sp, DJ-12]	5,50E-87	85.9	AAF16410	
(Continues)											

(Continues)

Table 3. Continued

ORF	Contig	Name	Position	#AA	Predicted function	hmmscan		BLASTp		Accession n°
						Description		Description (Identifier)	E-value	
7	L S M A contig008	vanB	5–361	118	Dioxygenase reductase	2Fe–2S iron–sulfur cluster binding domain		Vanillate O-demethylase oxidoreductase [Ottowia thiooxydans]	2.19E-66	WP_028605018
36	contig021	ccdC	1 195–1 941	248	Chlorocatechol 1,2-dioxygenase	Dioxygenase		Chlorocatechol 1,2-dioxygenase [Variovorax sp. WDL1]	0	AGF25473
41	contig021	ccdF	4304–5317	337	Alcohol dehydrogenase	Iron-containing alcohol dehydrogenase		maleyl acetate reductase [Variovorax sp. WDL1]	0	AGF25469
42	contig021	ccdD	5 280–6 497	405	Chloromuconate cycloisomerase	Enolase C-terminal domain-like		Chloromuconate cycloisomerase [Variovorax sp. WDL1]	0	AGF25468
43	contig021	ccdE	6490–7188	1320	Dienelactone hydrolase	Dienelactone hydrolase family		Dienelactone hydrolase [Variovorax sp. WDL1]	1.08E-169	100 AGF25467
50	contig022	phyH	2 704–1 838	288	Phytanoyl-CoA dioxygenase	Phytanoyl-CoA dioxygenase (PhyH)		Phytanoyl-CoA dioxygenase [Variovorax sp. WDL1]	0	100 AGF25453
53	contig022	hylA	6 070–4 322	582	Linuron hydrolase	Amidohydrolase family 3		Linuron hydrolase HylA [Variovorax sp. PBS-H4]	0	100 AGF25501
54	contig023	dcaA1	1 060–416	214	Dichloroaniline dioxygenase small subunit	Ring-hydroxylating β -subunit		Aniline dioxygenase small subunit [Variovorax sp. WDL1]	6.42E-139	100 AGF25495
55	contig023	dcaA2	1 299–1 057	80	Dichloroaniline dioxygenase large subunit [partial]			Aniline dioxygenase large subunit [Variovorax sp. WDL1]	1.41E-49	100 AGF25496
64	contig032	dcaT	1153–1034	39	Aniline dioxygenase (amino group transferase unit) [partial]			TdnT [Pseudomonas putida]	1.10E-18	97.4 YP_009077021
67	contig033		786–1 574	262		Amidohydrolase family 2		Hypothetical protein [Ottowia thiooxydans]	5.27E-149	80.4 WP_036595361
69	contig033		2599–3666	360	Alcohol dehydrogenase	Iron-containing alcohol dehydrogenase		Maleylacetate reductase [Ottowia thiooxydans]	0	74.4 WP_028604005
82	contig038		804–235	189	Monooxygenase	FAD binding domain		Putative monooxygenase [uncultured bacterium]	4.66E-60	53.1 ACV30051
86	contig041		1 705–458	415	Dioxygenase α -subunit	Rieske [2Fe–2S] domain		Hypothetical protein, partial [Ottowia thiooxydans]	0	100 WP_036596184
88	contig042		1 767–436	443		γ -glutamyltranspeptidase		γ -glutamyltransferase [Candidatus Solibacter usitatus Ellin6076]	0	65.7 ABJ85657
157	contig073		37–375	112	Reductase	2Fe–2S iron–sulfur cluster binding domain		Electron transfer protein [Variovorax sp. WDL1]	2.00E-70	99 WP_032490114

Functions were assigned according to detected Hidden Markov Models (HMM) with hmmscan and BLASTp protein similarities found in the predicted protein sequence of each ORF. Abbreviations: AA: Amino acid; AA ID: Amino acid identity.

contig 85 in BPS Kortrijk, 52%; contig 186 in BPS Kortrijk, 48%), osmosis regulatory channel (contig 53 in L SM A, 62%) mercury resistance regulator MerR (contig 260 in BPS Kortrijk, 46%) and DNA repair (contig 275 in BPS, UvrB, 81%). A large part of the ORFs represented unknown proteins. Some contigs even consisted of only ORFs to which no function could be assigned (either being unknown or showing similarity to ORFs with unknown function).

Genetic organization and synteny of metagenomic LR-PCR amplicons

Mega-BLAST searches performed on the complete LR amplicon contigs against the NCBI nr-database provided insight into gene synteny between the LR-PCR contigs and available genomic information as well as between the different LR-PCR contigs. Some of these contigs encoded protein functions identified by the previous BLASTp analysis, and other short contigs carried incomplete genes. Forty-eight BPS Kortrijk contigs showed high synteny with more than 95% nt similarity over the full length with a sequence in the NCBI nr-database, of which 29 were plasmid-borne. Similarly, several contigs recovered from L SM A (24 contigs; 11 plasmid-borne) and BPS pristine (2 contigs; 1 plasmid-borne) showed significant synteny and high nt similarity with reported bacterial genome regions and especially plasmid regions. Identified plasmid-borne sequences included both plasmid backbone gene clusters and accessory gene clusters. Plasmid regions were particularly central control regions containing IncP-1 plasmid maintenance functions showing complete synteny with at least one reported IncP-1 plasmid of either the IncP-1 β 1 or IncP-1 ϵ group (Supporting Information Fig. S9). Similarly, the contigs that carried the *hylA* gene for linuron degradation (contig 22 in L SM A and contig 290 in BPS Kortrijk as well the contigs carrying the dichloroaniline degradation genes (contigs 294 and 250 in BPS Kortrijk; contig 23 in L SM A) and chlorocatechol degradation gene clusters (contig 117 in BPS Kortrijk and contig 21 in L SM A) showed complete synteny (with 99% nt similarity) with corresponding IS1071 bordered (at both sites) catabolic regions in the linuron degrading *Variovorax* sp. WDL1 (Fig. 4A; Supporting Information Table S1). Moreover, also contigs 8 and 204 of BPS Kortrijk as well as contigs 39, 26 and 6 of L SM A corresponded to these regions (Fig. 4A; Supporting Information Table S1). Similarly, contig 260 carrying *atzA* showed complete synteny with the corresponding atrazine catabolic region of IncP-1 β 1 plasmid pADP-1, which is bordered by IS1071 elements at both ends (Fig. 4B; Supporting Information Table S1). Contig 7 of BPS Kortrijk was almost identical in nt sequence and organization with a IS1071 bordered

(at one site) uncharacterized catabolic gene region on plasmids pAKD33 and pAKD15 (Supporting Information Table S1; Fig. 4C) encoding putative alpha and beta ring-hydroxylating dioxygenase subunits (ORF26 and ORF27 on contig 7). Another part of the catabolic gene cluster located on pAKD15 was retrieved on contig 176 of BPS Kortrijk and contains two other putative dioxygenase genes (ORF348 and ORF351). Besides the contigs that encoded linuron hydrolysis, dichloroaniline degradation and chlorocatechol degradation, only two other regions were identified with substantial synteny between the BPS Kortrijk and L SM A amplicons. Contigs 41 and 5 showed nt identity and synteny with contig 276 of BPS Kortrijk while contigs 314 and 70 of BPS Kortrijk showed synteny with contig 33 of L SM A. These regions encoded for a putative dioxygenase (BPS Kortrijk, contig 276; L SM A, contig 41) and putative amidohydrolase (BPS Kortrijk, contig 314; L SM A, contig 33; Supporting Information Fig. S4; Table S2).

Synteny relationships with published genomes but based on less nt homology was explored by checking the BLASTp comparison results against the nr protein sequences for ORF clusters that show identical gene organizations as those found in the LR-PCR amplicons. Results are compiled in Supporting Information Table S3 while examples are shown in Supporting Information Fig. S8 for some contigs recovered from BPS Kortrijk, i.e. contig 5 containing a cobalt-containing nitrile hydratase cluster, contig 26 containing an unknown ABC-transporter system and contig 44 containing a complete urea transport and hydrolysis cluster. Interestingly, while IS1071 bordered at least one of the junctions in syntenic regions that showed more than 95% nt identity and near perfect synteny, this often was not the case for the syntenic gene clusters that showed less nt sequence identity. A substantial fraction (around 60%) of those however showed the presence of nearby IS elements (Supporting Information Table S3).

Pesticide degradation and mineralization capacity

Since genes involved in atrazine and linuron were recovered from the BPS samples, samples taken from the BPS Kortrijk at different time points during the agricultural season of 2011 were assessed for linuron mineralization and atrazine degradation. As a control, pristine BPS material was tested. All BPS Kortrijk samples showed mineralization of linuron and degradation of atrazine, while the control pristine BPS samples did not (Supporting Information Figs. S5 and S6).

Discussion

Accessory regions of IS1071 composite transposons were captured from complex microbial communities

Fig. 4. Nucleotide sequence similarity and synteny between selected contigs derived from the NGS of LR-PCR amplicons and relevant sequences in the nr-database. Sequence similarities with 99%–100% nucleotide identity as determined using BLASTn are shown by shaded regions.

A. Comparison of retrieved contigs of BPS Kortrijk and L SM A and catabolic regions of *Variovorax* sp WDL1 (accession numbers KC146403, KC146404 and KC146405). Key gene functions for the degradation of linuron are highlighted: initial linuron hydrolase, *hylA* (green), 3,4-dichloroaniline dioxygenase subunits, *dcaA1A2* (orange) and the catechol 1,2-dioxygenase, *ccdC* (magenta).

B. Comparison of contig 260 of BPS Kortrijk with the IS1071 composite transposon of IncP-1 plasmid pADP-1 containing *atxA* (blue) responsible for the initial dechlorination step in the bacterial degradation of atrazine in *Pseudomonas* sp. ADP1.

C. Comparison of contigs 7 and 176 recovered from BPS Kortrijk with uncharacterized catabolic gene clusters present on plasmids pAKD15 and pAKD33. The region on pAKD33 includes genes encoding a putative alpha and beta ring-hydroxylating dioxygenase subunit corresponding to ORF26 and ORF27 (yellow) located on contig 7. The region on pAKD15 contains two additional putative dioxygenase related genes corresponding to ORF348 and ORF351 in contig 176 (purple).

without cultivation step to further elucidate the ecological function of IS1071 beyond the information known from cultured isolates. To this end, we developed a high sensitivity LR-PCR method that recovers long DNA fragments from high molecular weight eDNA. By applying this method on environmental samples with a record of regular pesticide exposure, we were able to capture known and potential novel xenobiotic catabolic gene functions,

giving support to IS1071 being involved in the genetic adaptation of natural microbial communities.

LR-PCR to recover accessory regions of IS1071 composite transposons: opportunities and limitations

An LR-PCR method that combines selectivity/specificity and sensitivity to recover accessory regions of IS1071

composite transposons was successfully developed. The method selectively recovered regions located between two IS1071 elements based on primers directed outward of IS1071. The LR-PCR primers were tailored to target two IS1071 subtypes, as the existence of two IS1071 subtypes was proposed by Ma *et al.* (2007) based on the analysis of a metal resistance/catabolic gene cluster on plasmid pCNB1. Our analysis of current available IS1071 sequences showed that the IS1071 subtype 2 group represents up to 10% of the IS1071 sequences and hence that subtype 2 is an important group that needs to be covered. Moreover, the same composite transposon can be bordered by two subtypes. Indeed, 10 of the 11 reported gene clusters that are flanked by IS1071 subtype 2 at one site are flanked by a subtype 1 IS1071 element at the other site (Boon *et al.*, 2001; Dejonghe *et al.*, 2002; Bers *et al.*, 2011; Ma *et al.* 2007). Sequence analysis of the LR-PCR amplicons provided immediate proof of principle of selectivity of the method. Of the contigs 28% contained partial IS1071 IR sequences at least on one of the borders, implying that the contigs were truly carried by IS1071 based composite transposons. Moreover, contigs bordered by both IS1071 subtype 1 and subtype 2 were recovered. IS1071 subtype 1 was the dominant subtype in the three examined ecosystems which agrees with its dominance among reported IS1071 elements in the nt database. However, IS1071 subtype 2 elements were recovered in both the BPS Kortrijk and L SM A samples providing evidence for its ecological relevance. Interestingly, potentially a third IS1071 subtype was identified in this study showing most resemblance with subtype 1 IS1071 (87% nt identity).

The size of the recovered amplicons is an important item in the method since IS1071 composite transposon accessory regions of up to 43 kb have been reported. Amplicons of up to 20 kb were efficiently recovered using our LR-PCR method. Increasing the elongation time resulted into larger amplicons but also into the amplification of unwanted plasmid backbone regions when plasmid DNA carrying IS1071 composite transposons was used as a template. As such, taking into account that IS1071 composite transposons are often carried by plasmids, the elongation time was set to 16 min, limiting the accessory regions under study to 20 kb. Analysis of the lengths of accessory regions bordered by IS1071 elements in the NCBI database suggests that around 34% was excluded, assuming no bias in the database. However, even with the current protocol, 5.5% of the contigs consisted of plasmid backbone. Even though these amplicons are unintended, they also provide interesting information about the plasmid replicons that are carrying them. We found that the recovered plasmid backbone regions especially concerned regions from IncP-1 plasmids of the IncP-1 β 1 and IncP-1 ϵ subgroups (Tsuda

et al., 2014). This is in agreement with IncP-1 plasmids found so far in isolates, confirming that these two IncP-1 subgroups are important carriers of IS1071 composite transposons. It further suggests that at least a fraction of the IS1071 and its associated accessory genes are located on plasmids and in particular on IncP-1 plasmids. IncP-1 plasmids have two major insertion hot spots for accessory genes (Supporting Information Fig. S10) including IS1071 composite transposons, i.e., in between the origin of replication (*oriV*) and the gene encoding the replication initiation protein (*trfA*) and in between the *trb*-operon and *traC* (Sota *et al.*, 2007). Therefore, backbone regions based on those insertion sites were expected to appear in our LR-PCR amplicons, but this was not the case. Instead, the central control region (*re/E-kfrA*), covering *klcBA*, *kleFEBA* and *korBAC*, was amplified most frequently. In IncP-1 β plasmid pB4, the location of the accessory genes deviates from other IncP-1 plasmids and is between *traM* and *kfrC* (Tauch *et al.*, 2003). Amplification of the central control region can be explained by the simultaneous presence of IS1071 in the insertion sites *traM-kfrC* and *oriV-trfA* (Supporting Information Fig. S10). Hence, the frequent recovery of the central control region of IncP-1 might suggest that *traM-kfrC*, is a more important hot spot of insertion in IncP-1 plasmids than known from bacterial isolates.

The detection limit of the method of 10⁴ copies per gram of background matrix is within the sensitivity range of endpoint PCR targeting individual genes (Leys *et al.*, 2004). As such, although the method will suffer from drawbacks inherent to PCR like disclosing rare targets (Murray *et al.*, 1996) and more efficient amplification of smaller regions compared to larger ones (Williams *et al.*, 2006), it combines the ability to selectively recover long DNA fragments from eDNA with the high sensitivity of classic PCR. Moreover, agarose gel electrophoresis of the amplicons provides a first snapshot of the recovered amplicon sizes and hence provides a tool to rapidly examine differences in diversity between replicate samples and environments and to select samples for further NGS. Overall, the method extends the metagenomic mobilome toolbox that includes other molecular approaches like direct plasmid extraction combined with NGS or replicon typing by PCR-NGS (Sentchilo *et al.*, 2013; Dealtry *et al.*, 2014b).

A large fraction of the gene functions on the LR-PCR recovered IS1071 associated accessory regions encode for catabolic enzymes

Sequence analysis of the contigs showed that the IS1071 composite transposons accessory genes recovered from the pesticide-treated environments often have a catabolic nature. KEGG analysis identified mainly lower

pathway catabolic gene functions while HMMR and BLASTp led to the discovery of putative upstream catalytic functions in xenobiotic catabolic pathways (i.e. *atzA* and *hylA*). This observed discrepancy between analysis methodologies could primarily be attributed to the absence of plasmid-encoded genes in the BlastKOALA database (K. Morishima, 2016, pers. comm.). Both central and peripheral catabolic pathways included functions that could be directly linked to known pesticide biodegradation functions like *hylA*, *dca*-genes (Bers *et al.*, 2013a), *atzA* (de Souza *et al.*, 1998) and *tfdA* (Fukumori and Hausinger, 1993). *hylA/dca* are genes involved in the biodegradation of linuron, *atzA* in the biodegradation of atrazine, and *tfdA* in the biodegradation of chlorinated phenoxyacetic acids like 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA). Degradation of atrazine and mineralization of linuron was shown in the BPS samples while mineralization of linuron was previously shown in the L SM A sample (Bers *et al.*, 2013b), hence linking observed genotype with a community phenotype. The detection of the *hylA/dca*-genes corroborates previous observations in both L SM A and another BPS environment, either using targeted PCR, exogenous plasmid isolation or cultivation-dependent techniques (Horemans *et al.*, 2016; Bers *et al.*, 2013a; Dealtry *et al.*, 2016; Martini *et al.*, 2016). Interestingly, all three genotypes could also be linked to occurrence of the respective pesticide substrates the samples were exposed to, i.e. linuron in case of L SM A, and linuron and MCPA/2,4-D in case of BPS Kortrijk. Atrazine has been banned for use since many years but *atzA* can be linked to terbuthylamine, another triazine compound with structure highly similar to atrazine, present in the wastewater (Caracciolo *et al.*, 2010). Several other catabolic gene functions were recovered, including amidohydrolases and dioxygenases, which are likely involved in, but could not be directly linked to the degradation of a particular xenobiotic substrates. In BPS Kortrijk, besides triazine compounds, chlorinated phenoxyacetic acids and linuron, at least 33 other pesticides representing a wide range of chemical structures, were applied (Supporting Information Table S4) (Dunon *et al.*, 2013; Dealtry *et al.*, 2014b). The recovered catabolic accessory genes might thus play a role in the degradation of these compounds, for which the degradation pathways are often not known. Interestingly, two enzymes catalysing the conversion of nitriles (on contigs 5 and 44) were identified while the pesticides applied in the BPS included compounds containing nitrile groups like chlorotalonil and λ -cyhalothrin. In case of L SM A, the enrichment of unknown catabolic genes was clearly due to the application of linuron since no LR-PCR amplicons were recovered when no linuron was applied or after linuron application had been ceased for a longer period. The

absence of LR-PCR amplicons and hence reduced abundance of IS1071 composite transposons below the detection limit in the latter control samples is in accordance with the findings of Bers *et al.* (2013b) and Horemans *et al.* (2016), observing both significant drops (factor 100–1000 till close to the PCR detection limit) in the abundance of the linuron hydrolytic gene functions *libA* and *hylA* concomitant with a drop in the linuron mineralization efficiency. It remains unclear whether these 'unknown' degradative genes in the L SM A environment also contribute to the degradation of linuron by representing alternative catabolic routes, or whether they are merely gene clusters hitchhiking with linuron degrading bacteria that contained, for instance, *hylA* (Horemans *et al.*, 2016).

The high incidence of catabolic genes mirrors the selective pressure in the studied environments

The impressively high incidence of (relevant) catabolic genes in association with IS1071 in both pesticide-treated environments shows that catabolic genes combined with IS1071 are highly enriched in such habitats. Moreover, their presence mirrors the major pollution these environments experienced, i.e. the application of organic xenobiotics, and hence mirrors the environmental selection. This is further accentuated by (i) the much higher diversity of IS1071 associated catabolic gene functions found in BPS Kortrijk compared to L SM A, consistent with exposure to a higher variety of pesticides in BPS Kortrijk compared to L SM A receiving only linuron, (ii) the higher incidence of IS1071 associated catabolic genes in the pesticide-exposed environments compared to the controls and (iii) as illustrated above the direct link between several of the recovered catabolic functions and the pesticides to which the environments were exposed to. Our data thus strongly suggest that the catabolic IS1071 composite transposons were under strong selection from the organic pollutants, allowing their bacterial hosts to use these compounds as a carbon source or at least detoxify them. Currently, it is not clear whether this community adaptation involves simply enrichment of strains carrying the IS1071 associated genes, HGT or novel pathway construction on site. The LR-PCR approach allowed to have a cultivation-independent glance at the genetic context of catabolic genes in the microbial communities. The gene regions of the contigs that include *hylA*, *dca* and *atzA* showed remarkable nt identity and synteny with previously reported isofunctional gene regions bordered by IS1071 elements in bacterial strains (Bers *et al.*, 2011, 2013a; Martinez *et al.*, 2001) suggesting that the genetic context of these genes is quite conserved in the environment and apparently linked to IS1071. Therefore, their increased presence

might be primarily linked with the growth of the original hosts or new hosts after gene transfer. On the other hand, several contigs showed partial but remarkable synteny with gene clusters in other organisms, which often did not contain nearby IS1071. This might indicate that they do not necessarily move together with IS1071 and hence were only recently integrated into an IS1071 carrying constellation. The integration might have been promoted by other IS elements that were often detected in the contigs. Many of the known IS1071 composite transposons have large accessory regions that also harbour other transposases. IS elements often associated with IS1071 are IS21 and IS66 (Król *et al.*, 2012). IS21 was predominantly present in the recovered contigs and IS66 was also frequently detected. Interestingly, IS21 was often reoccurring in the sequences from the NCBI nt database that show significant synteny with gene clusters retrieved from the LR-PCR amplicons (Supporting Information Table S3). Furthermore, most of the organisms showing synteny were β -proteobacteria, which appear to be the preferential taxon for transposition of IS1071 among the proteobacteria (Sota *et al.*, 2006). Whether or not the catabolic genes were conserved into an IS1071 constellation or were recently captured, their association with IS1071 make them prone to HGT and hence makes them a common benefit to the local microbial community. Indeed, even when originally present on a non-transferable replicon, the composite transposon might transpose onto more promiscuous replicons like conjugative plasmids (Fulthorpe and Wyndham, 1992). The promiscuous IncP-1 plasmids that were also enriched in BPS and linuron-treated agricultural soils (Dunon *et al.*, 2013) make such an interesting vehicle for HGT of those IS1071 composite transposons, as was recently shown by the identification of *hylA* containing IncP-1 plasmids in the same BPS environment examined in the current study (Dealtry *et al.*, 2016).

The recovered IS1071 accessory regions contain a variety of non-catabolic gene functions and unknown genes

Up to now, only two IS1071 based composite transposons were reported carrying gene functions that do not specify organic xenobiotic degradation, containing respectively an antibiotic resistance (Schlüter *et al.*, 2003) and a metal resistance operon (accession number CP001644). Both can be considered as responsive to environmental selection. Interestingly, many genes were recovered with functions deviating from classical adaptive functions including genes that encode proteins that show similarity to proteins involved in membrane transport, ribosomal translation, signal transduction and cellular community, indicating that IS1071 can be involved in

distributing other gene functions. These other gene functions are often present on contigs containing at least one putative catabolic gene function and hence they might be involved in optimizing the xenobiotic biodegradation function carried by the same transposon. Otherwise, they might improve the bacterial host's fitness in another yet unknown way. On the other hand, often the similarity for various non-catabolic proteins is below 40% when compared with a known protein sequence and therefore, it remains uncertain that these proteins perform the same function. An unexpected observation was the recovery of clusters of r-protein genes from agricultural soil sample L SM A. Those genes seem to originate from *Acidobacteria* which are common soil bacteria (Kielak *et al.*, 2016). The two corresponding contigs do not contain IS1071 remains at their sequence ends and therefore, at this stage, we cannot rule out that their presence would be due to unspecific amplification due to a high number of *Acidobacteria* in the L SM A soil sample. However, this is unlikely, since, besides the r-protein gene clusters, no other genes associated with *Acidobacteria* were recovered. The association of r-protein genes with IS1071 would make them prone to HGT. HGT gene transfer of informational genes like those encoding for r-proteins is considered unlikely. On the other hand, some phylogenetic studies show some unexpected tree topologies that (Makarova *et al.*, 2001) suggest a role of HGT in the evolution of r-proteins.

In addition to genes encoding for non-catabolic proteins, a large fraction (30–40%) of the genes in the recovered contigs could still not be assigned a known function, opening a window for an even broader range of enzyme functions to be associated with IS1071 elements. These results are reminiscent of a study by Sentchilo *et al.* (2013) that examined the plasmid metagenome from wastewater. These authors identified plasmid accessory genes involved in membrane transport and stress relief as well as a large number of unknown genes. As suggested by Sentchilo *et al.* (2013) for plasmids, our results suggest that IS1071 is a driving force for innovation of genetic information in bacteria (Sentchilo *et al.*, 2013).

In conclusion, our LR-PCR approach extends observation from cultured isolates and shows that IS1071 is an omnipresent carrier of xenobiotic degradative functions in complex communities of pesticide impacted environments and might contribute to the environmental distribution and evolution of such genes as a response to pesticide pollution. The data can be further used as starting point to improve our understanding of the pesticide biodegradation functions in pesticide-contaminated ecosystems including the identification of new pathways and enzymes, of the environmental dynamics and abundance of IS1071 and its subtypes and of the mechanism of gene recruitment by IS1071 as well distribution of IS1071

based composite transposons in microbial communities. Moreover, LR-PCR can be a powerful method to understand the ecological role of IS1071 in environmental settings in bacterial adaptation to environmental stressors different from organic pollution or sudden changes. It can also be used as a tool to mine interesting rare locally adaptive gene functions directly from the environment.

Experimental procedures

Plasmids used in this study

Plasmids carrying IS1071 composite transposons used in this study are presented in Table 1 and were propagated in various hosts. Corresponding cultivation media and conditions of the hosts are presented in Supporting Information.

Chemicals used in this study

Atrazine [2-chloro-4-ethylamine-6-isopropylamino-S-triazine] (99.5%) was purchased from Sigma Aldrich (Belgium) and [phenyl-U-14C] linuron (627 MBq mmol⁻¹, radiochemical purity >95%) from Izotop, Hungary.

Environmental samples

Environmental samples used in this study originated from an artificially composed BPS matrix (BPS pristine), from the matrix of a BPS in operation at a farm near Kortrijk, Belgium (BPS Kortrijk), and from a previously reported agricultural soil microcosm (SM) experiment. The non-pesticide treated artificial BPS matrix samples were composed of materials that are used in BPS and consisted of 25 vol% straw, 25 vol% coco chips and 50 vol% flower soil. The BPS Kortrijk operational conditions and details about the BPS Kortrijk sampling procedure were described before (Dealtry *et al.*, 2014b). Briefly, the BPS (length: 20 m, width: 1.2 m) was divided into four equal sampling sections. For each section, 12 cores (Ø4 cm; 10 cm depth) were collected randomly, mixed and sieved. Samples were collected before start-up in March 2011, during the spraying season in July and September of the agricultural season of 2011 and just after the seasonal operational shut down of the BPS in December 2011. Supporting Information Table S4 provides a list of the active substances present in the wastewater during the agricultural season 2011. Actual pesticide concentrations though are unknown and variable over time. The agricultural soil SM experiment was described before by Bers *et al.* (2013b) and aimed at assessing the response of *Variovorax* and its linuron hydrolysis gene *libA* to linuron application in agricultural soil. Details about its setup and operation can be found in Bers *et al.* (2013b).

Briefly, SMs were glass columns filled with soil originating from an agricultural field with a history of linuron treatment. The SM experiment included two different treatments in triplicate: (i) irrigation with sterile tap water (triplicate microcosms 'W SM D', 'W SM E' and 'W SM F') and (ii) irrigation with sterile tap water containing 60 mg l⁻¹ linuron (triplicate microcosms 'L SM A', 'L SM B' and 'L SM C'). During the experiment, the treatment conditions changed for some replicate microcosms. Most importantly for this study, between days 285 and 491, the supply of linuron in microcosm 'L SM B' was replaced by adding tap water without linuron in order to confirm observed previously observed responses to linuron application/non-application. At days 0, 28, 110, 149, 245 and 491, the soil was sampled from the microcosms for analysis. In this study, only the samples taken at day 491 were used for LR-PCR analysis.

Plasmid DNA extraction

Large plasmid DNA from bacterial cultures was isolated using an adapted alkaline lysis method (Sambrook and Russell, 2001) as reported in Supporting Information.

eDNA extraction

eDNA from the BPS Kortrijk samples for qPCR purposes was extracted using the FastDNA SPIN kit for Soil (MP Biomedicals) and further purified with the GENE-CLEAN Spin kit (Dealtry *et al.*, 2014b). For all samples, high molecular weight eDNA for LR-PCR purposes was extracted from 300 mg wet weight material using the Meta-G-Nome™ DNA isolation kit from Epicentre with a slightly adapted protocol as reported in Supporting Information.

Real-time PCR

Quantification of IS1071 *tnpA* was performed by real-time PCR as described (Dunon *et al.*, 2013). Significant differences in relative abundances between samples were examined by one-way analysis of variance (ANOVA; $p < 0.05$) and *post hoc* Tukey test using SPSS (IBM, Armonk, NY).

Long range PCR

A database of 91 complete and incomplete IS1071 sequences (>1.5 kb; Accession numbers are in Supporting Information) was obtained by specific BLASTn searches and manually curated. Sequences were aligned with CLUSTAL X (Larkin *et al.*, 2007) and a phylogenetic maximum likelihood tree was generated using MEGA6 (Tamura *et al.*, 2013). Degenerate primers, IS1071_LR_1 (5'-ACAGGGTGGCCACCTCCAASGCCYGAAC-3') and

IS1071_LR_2 (5'-GAGCTGCTGATGGTCTAYGCCGG-CATCAT-3'), were designed directing outwards in a highly conserved region of the *tnpA* gene. The IS1071 LR-PCR was performed in a volume of 25 µl containing 1 U Long PCR enzyme mix (Thermo Scientific, Germany) and 1× Long PCR buffer, 200 µM of each dNTP, 0.1 mg ml⁻¹ BSA, 4% (v/v) DMSO and 0.4 µM and the primers IS1071_LR_1 and IS1071_LR_2. The LR-PCR included an initial denaturation of 2 min at 94 °C, 10 cycles of 20 s at 94 °C and elongation of 16 min at 68 °C, followed by 25 cycles in which the elongation time was increased every cycle with 5 s and ended with an elongation step of 10 min at 68 °C. PCR products were separated by agarose gel electrophoresis (0.8%) in Tris-acetate/EDTA buffer and visualized using 1× GelRed™ (Biotium, Fremont, CA).

Southern blot hybridization

Southern blot hybridization of LR-PCR products using a *dcaQ* gene fragment as a probe was performed as described in Supporting Information.

Pesticide mineralization and degradation assays

¹⁴C-linuron mineralization assays were performed on 200 mg (wet weight) of the environmental sample in MMO medium (Dejonghe *et al.*, 2002) in triplicate and cumulative linuron mineralization curves composed as described (Sniegowski *et al.*, 2011a, 2011b). Atrazine degradation assays were performed in triplicate in 75 ml Erlenmeyer flasks containing 1 g (wet weight) of BPS material suspended in 25 ml MMO that contained 15 mg l⁻¹ atrazine. Abiotic controls containing sterilized BPS material (three times autoclaved), were included. The flasks were incubated on a horizontal shaker (125 rpm) at 20 °C in the dark for 13 days and 700 µl samples taken regularly, acidified with 1 vol% HCl (5 M) and centrifuged at 14 000 × g for 10 min. Atrazine concentrations were quantified in the supernatant as reported previously (Cheyns *et al.*, 2012).

Sequencing and annotation

LR-PCR amplicons were ethanol precipitated in 10 mM Tris-HCl/EDTA [pH 8.0] and used for NGS by BGI Hong Kong. Paired-end sequencing (2 × 100 bp) was performed with the HiSeq 2000 (Illumina) platform. Two different runs were conducted using an insert library size of 200 bp for the amplicons of BPS Kortrijk and L SM A and 500 bp for the amplicons of BPS pristine. Velvet 10.1 and Meta-Velvet (Zerbino and Birney, 2008) was used for reads assembly (see Supporting Information) and Bowtie 2 (version 2.2.5) for mapping back the reads on the contigs. Gene prediction

and annotation was performed by RAST (Aziz *et al.*, 2008), HMMER (Jeffries *et al.*, 2016), BLASTp (Ramsay *et al.*, 2000) and Mega-BLAST (Altschul *et al.*, 1997). KEGG Orthologies (KOs) were assigned by BlastKOALA (KEGG Orthology and Links Annotation) using the species_prokaryotes KEGG GENES database. Classification in functional categories was performed using the reconstruct pathway output (Kanehisa *et al.*, 2016).

Accession numbers

Raw sequences are deposited in the Sequence Read Archive under the BioProjects: PRJNA278476 (BPS Kortrijk), PRJNA279102 (BPS Pristine) and PRJNA273666 (L SM A). Assembled and annotated contigs are deposited in the GenBank database with following accession numbers: KY206930-KY207245 (BPS Kortrijk), KY207246-KY207262 (BPS Pristine) and KY207263-KY207335 (L SM A).

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References

- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.
- Aziz, R. K., Bartels, D., Best, A., DeJongh, M., Disz, T., Edwards, R. A., *et al.* (2008) The RAST server: rapid annotations using subsystems technology. *BMC Genomics* **9**: 75.
- Bers, K., Batisson, I., Proost, P., Wattiez, R., De Mot, R., and Springael, D. (2013a) HylA, an alternative hydrolase for initiation of catabolism of the phenylurea herbicide linuron in *Variovorax* sp. strains. *Appl Environ Microbiol* **79**: 5258–5263.
- Bers, K., Leroy, B., Breugelmans, P., Albers, P., Lavigne, R., Sørensen, S. R., *et al.* (2011) A novel hydrolase identified by genomic-proteomic analysis of phenylurea herbicide mineralization by *Variovorax* sp. strain SRS16. *Appl Environ Microbiol* **77**: 8754–8764.
- Bers, K., De Mot, R., and Springael, D. (2013b) *In situ* response of the linuron degradation potential to linuron application in an agricultural field. *FEMS Microbiol Ecol* **85**: 403–416.
- Boon, N., Goris, J., De Vos, P., Verstraete, W., and Top, E. M. (2001) Genetic diversity among 3-chloroaniline- and aniline-degrading strains of the *Comamonadaceae*. *Appl Environ Microbiol* **67**: 1107–1115.
- Caracciolo, A. B., Fajardo, C., Grenni, P., Saccà, M. L., Amalfitano, S., Ciccoli, R., *et al.* (2010) The role of a

- groundwater bacterial community in the degradation of the herbicide terbutylazine. *FEMS Microbiol Ecol* **71**: 127–136.
- Cheyens, K., Calcoen, J., Martin-Laurent, F., Bru, D., Smolders, E., and Springael, D. (2012) Effects of dissolved organic matter (DOM) at environmentally relevant carbon concentrations on atrazine degradation by *Chelatobacter heintzii* SalB. *Appl Microbiol Biotechnol* **95**: 1333–1341.
- Dealtry, S., Ding, G. C., Weichelt, V., Dunon, V., Schlüter, A., Martini, M. C., et al. (2014a) Cultivation-independent screening revealed hot spots of IncP-1, IncP-7 and IncP-9 plasmid occurrence in different environmental habitats. *PLoS One* **9**: e89922.
- Dealtry, S., Holmsgaard, P. N., Dunon, V., Jechalke, S., Ding, G.-C., Krögerrecklenfort, E., et al. (2014b) Shifts in abundance and diversity of mobile genetic elements after the introduction of diverse pesticides into an on-farm biopurification system over the course of a year. *Appl Environ Microbiol* **80**: 4012–4020.
- Dealtry, S., Nour, E. H., Holmsgaard, P. N., Ding, G.-C., Weichelt, V., Dunon, V., et al. (2016) Exploring the complex response to linuron of bacterial communities from biopurification systems by means of cultivation-independent methods. *FEMS Microbiol Ecol* **92**: 1–12.
- Dejonghe, W., Goris, J., Dierickx, A., Dobbeleer, V., Crul, K., De Vos, P., et al. (2002) Diversity of 3-chloroaniline and 3,4-dichloroaniline degrading bacteria isolated from three different soils and involvement of their plasmids in chloroaniline degradation. *FEMS Microbiol Ecol* **42**: 315–325.
- Dennis, J. J. (2005) The evolution of IncP catabolic plasmids. *Curr Opin Biotechnol* **16**: 291–298.
- de Souza, M. L., Seffernick, J., Martinez, B., Sadowsky, M. J., and Wackett, L. P. (1998) The atrazine catabolism genes *atzABC* are widespread and highly conserved. *J Bacteriol* **180**: 1951–1954.
- Drolet, M., Zanga, P., and Lau, P. C. K. (1990) The mobilization and origin of transfer regions of a *Thiobacillus ferrooxidans* plasmid: relatedness to plasmids RSF1010 and pSC101. *Mol Microbiol* **4**: 1381–1391.
- Dunon, V., Sniegowski, K., Bers, K., Lavigne, R., Smalla, K., and Springael, D. (2013) High prevalence of IncP-1 plasmids and IS1071 insertion sequences in on-farm biopurification systems and other pesticide-polluted environments. *FEMS Microbiol Ecol* **86**: 415–431.
- Fang, H., Cai, L., Yang, Y., Ju, F., Li, X., Yu, Y., et al. (2014) Metagenomic analysis reveals potential biodegradation pathways of persistent pesticides in freshwater and marine sediments. *Sci Total Environ* **470–471**: 983–992.
- Fukumori, F., and Hausinger, R. P. (1993) *Alcaligenes eutrophus* JMP134 '2,4-dichlorophenoxyacetate monooxygenase' is an alpha-ketoglutarate-dependent dioxygenase. *J Bacteriol* **175**: 2083–2086.
- Fulthorpe, R. R., and Wyndham, R. C. (1992) Involvement of a chlorobenzoate-catabolic transposon, Tn5271, in community adaptation to chlorobiphenyl, chloroaniline, and 2,4-dichlorophenoxyacetic acid in a freshwater ecosystem. *Appl Environ Microbiol* **58**: 314–325.
- Halak, S., Basta, T., Bürger, S., Contzen, M., Wray, V., Pieper, D. H., et al. (2007) 4-Sulfomuconolactone hydrolases from *Hydrogenophaga intermedia* S1 and *Agrobacterium radiobacter* S2. *J Bacteriol* **189**: 6998–7006.
- Horemans, B., Bers, K., Romero, E. R., Juan, E. P., Dunon, V., De Mot, R., et al. (2016) Functional redundancy of linuron degradation in microbial communities in agricultural soil and biopurification systems. *Appl Environ Microbiol* **82**: 2843–2853.
- Iwasaki, W., and Takagi, T. (2009) Rapid pathway evolution facilitated by horizontal gene transfers across prokaryotic lineages. *PLoS Genet* **5**: e1000402.
- Jeffryes, M., Liakata, M., and Bateman, A. (2016) Crowd-sourcing protein family database curation. *CEUR Workshop Proc* **1747**: W29–W37.
- Kanehisa, M., Sato, Y., and Morishima, K. (2016) BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *J Mol Biol* **428**: 726–731.
- Kielak, A. M., Barreto, C. C., Kowalchuk, G. A., van Veen, J. A., and Kuramae, E. E. (2016) The ecology of *Acidobacteria*: moving beyond genes and genomes. *Front Microbiol* **7**: 744.
- Kobayashi, M., and Shimizu, S. (1998) Metalloenzyme nitrile hydratase: structure, regulation, and application to biotechnology. *Nat Biotechnol* **16**: 733–736.
- Król, J. E., Penrod, J. T., McCaslin, H., Rogers, L. M., Yano, H., Stancik, A. D., et al. (2012) Role of IncP-1 β plasmids pWDL7::rfp and pNB8c in chloroaniline catabolism as determined by genomic and functional analyses. *Appl Environ Microbiol* **78**: 828–838.
- Kumar, A., Trefault, N., and Olaniran, A. O. (2016) Microbial degradation of 2,4-dichlorophenoxyacetic acid: insight into the enzymes and catabolic genes involved, their regulation and biotechnological implications. *Crit Rev Microbiol* **42**: 194–208.
- Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., et al. (2007) ClustalW and ClustalX version 2.0. *Bioinformatics* **23**: 2947–2948.
- Layton, A. C., Sanseverino, J., Wallace, W., Corcoran, C., and Saylor, G. S. (1992) Evidence for 4-chlorobenzoic acid dehalogenation mediated by plasmids related to pSS50. *Appl Environ Microbiol* **58**: 399–402.
- Lee, M. H., Mulrooney, S. B., Renner, M. J., Markowicz, Y., and Hausinger, R. P. (1992) *Klebsiella aerogenes* urease gene cluster: sequence of *ureD* and demonstration that four accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*) are involved in nickel metallocenter biosynthesis. *J Bacteriol* **174**: 4324–4330.
- Ledger, T., Pieper, D. H., and González, B. (2006) Chlorophenol hydroxylases encoded by plasmid pJP4 differentially contribute to chlorophenoxyacetic acid degradation. *Appl Environ Microbiol* **72**: 2783–2792.
- Leys, N. M. E. J., Ryngaert, A., Bastiaens, L., Verstraete, W., Top, E. M., and Springael, D. (2004) Occurrence and phylogenetic diversity of *Sphingomonas* strains in soils contaminated with polycyclic aromatic hydrocarbons. *Appl Environ Microbiol* **70**: 1944–1955.
- Ma, Y. F., Wu, J. F., Wang, S. Y., Jiang, C. Y., Zhang, Y., Qi, S. W., et al. (2007) Nucleotide sequence of plasmid pCNB1 from *Comamonas* strain CNB-1 reveals novel genetic organization and evolution for 4-chloronitrobenzene degradation. *Appl Environ Microbiol* **73**: 4477–4483.
- Makarova, K. S., Ponomarev, V. A., and Koonin, E. V. (2001) Two C or not two C: recurrent disruption of Zn-ribbons, gene duplication, lineage-specific gene loss,

- and horizontal gene transfer in evolution of bacterial ribosomal proteins. *Genome Biol* **2**: RESEARCH 0033.
- Martinez, B., Tomkins, J., Wackett, L. P., Wing, R., and Sadowsky, M. J. (2001) Complete nucleotide sequence and organization of the atrazine catabolic plasmid pADP-1 from *Pseudomonas* sp. strain ADP. *J Bacteriol* **183**: 5684–5697.
- Martini, M. C., Wibberg, D., Lozano, M., Torres Tejerizo, G., Albicoro, F. J., Jaenicke, S., *et al.* (2016) Genomics of high molecular weight plasmids isolated from an on-farm biopurification system. *Sci Rep* **6**: 28284.
- Müller, T. A., Fleischmann, T., van der Meer, J. R., and Kohler, H.-P. E. (2006) Purification and characterization of two enantioselective alpha-ketoglutarate-dependent dioxxygenases, RdpA and SdpA, from *Sphingomonas herbicidovorans* MH. *Appl Environ Microbiol* **72**: 4853–4861.
- Murray, A. E., Hollibaugh, J. T., and Orrego, C. (1996) Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Appl Environ Microbiol* **62**: 2676–2680.
- Nojiri, H., Shintani, M., and Omori, T. (2004) Divergence of mobile genetic elements involved in the distribution of xenobiotic-catabolic capacity. *Appl Microbiol Biotechnol* **64**: 154–174.
- Ramsay, L., Macaulay, M., Degli Ivanisovich, S., MacLean, K., Cardle, L., Fuller, J., *et al.* (2000) A simple sequence repeat-based linkage map of barley. *Genetics* **156**: 1997–2005.
- Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd ed. New York: Cold Spring Harbor Lab Press.
- Schlüter, A., Heuer, H., Szczepanowski, R., Forney, L. J., Thomas, C. M., Pühler, A., *et al.* (2003) The 64 508 bp IncP-1 β antibiotic multiresistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1 β group. *Microbiology* **149**: 3139–3153.
- Sen, D., Yano, H., Suzuki, H., Król, J. E., Rogers, L., Brown, C. J., and Top, E. M. (2010) Comparative genomics of pAKD4, the prototype IncP-1 δ plasmid with a complete backbone. *Plasmid* **63**: 98–107.
- Sentchilo, V., Mayer, A. P., Guy, L., Miyazaki, R., Tringe, S. G., Barry, K., *et al.* (2013) Community-wide plasmid gene mobilization and selection. *ISME J* **7**: 1173–1186.
- Shin, K. A., and Spain, J. C. (2009) Pathway and evolutionary implications of diphenylamine biodegradation by *Burkholderia* sp. strain js667. *Appl Environ Microbiol* **75**: 2694–2704.
- Sniegowski, K., Bers, K., Van Goetem, K., Ryckeboer, J., Jaeken, P., Spanoghe, P., *et al.* (2011a) Improvement of pesticide mineralization in on-farm biopurification systems by bioaugmentation with pesticide-primed soil. *FEMS Microbiol Ecol* **76**: 64–73.
- Sniegowski, K., Bers, K., Ryckeboer, J., Jaeken, P., Spanoghe, P., and Springael, D. (2011b) Robust linuron degradation in on-farm biopurification systems exposed to sequential environmental changes. *Appl Environ Microbiol* **77**: 6614–6621.
- Sota, M., Kawasaki, H., and Tsuda, M. (2003) Structure of haloacetate-catabolic IncP-1 β plasmid pUO1 and genetic mobility of its residing haloacetate-catabolic transposon. *J Bacteriol* **185**: 6741–6745.
- Sota, M., Tsuda, M., Yano, H., Suzuki, H., Forney, L. J., and Top, E. M. (2007) Region-specific insertion of transposons in combination with selection for high plasmid transferability and stability accounts for the structural similarity of IncP-1 plasmids. *J Bacteriol* **189**: 3091–3098.
- Sota, M., Yano, H., Nagata, Y., Ohtsubo, Y., Genka, H., Anbutsu, H., *et al.* (2006) Functional analysis of unique class II insertion sequence IS1071. *Appl Environ Microbiol* **72**: 291–297.
- Sota, M., Yano, H., and Tsuda, M. (2008) Bacterial class II catabolic transposons. In *DNA Transposable Elements Research*, Yoshida, K., and Aoki, M. (eds). New York, NY: Nova Science Publishers, Inc, pp. 23–67.
- Suenaga, H. (2012) Targeted metagenomics: a high-resolution metagenomics approach for specific gene clusters in complex microbial communities. *Environ Microbiol* **14**: 13–22.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* **30**: 2725–2729.
- Tauch, A., Schlüter, A., Bischoff, N., Goesmann, A., Meyer, F., and Pühler, A. (2003) The 79,370-bp conjugative plasmid pB4 consists of an IncP-1 β backbone loaded with a chromate resistance transposon, the *strA-strB* streptomycin resistance gene pair, the oxacillinase gene *bla_{NPS-1}*, and a tripartite antibiotic efflux system of the resistance-nodulation-division family. *Mol Genet Genomics* **268**: 570–584.
- Top, E. M., and Springael, D. (2003) The role of mobile genetic elements in bacterial adaptation to xenobiotic organic compounds. *Curr Opin Biotechnol* **14**: 262–269.
- Tsuda, M., Ohtsubo, Y., and Yano, H. (2014) Mobile catabolic genetic elements in *Pseudomonads*. In *Biodegradative Bacteria: How Bacteria Degrade, Survive, Adapt, and Evolve*, Nojiri, H., Tsuda, M., Fukuda, M., and Kamagata, Y. (eds). Tokyo, Japan: Springer, pp. 83–103.
- van der Meer, J. R., Eggen, R. I. L., Zehnder, A. J. B., and de Vos, W. M. (1991) Sequence analysis of the *Pseudomonas* sp. strain P51 *tcb* gene cluster, which encodes metabolism of chlorinated catechols: evidence for specialization of catechol 1,2-dioxygenases for chlorinated substrates. *J Bacteriol* **173**: 2425–2434.
- Vedler, E., Vahter, M., and Heinaru, A. (2004) The completely sequenced plasmid pEST4011 contains a novel IncP1 backbone and a catabolic transposon harboring *tfd* genes for 2,4-dichlorophenoxyacetic acid degradation. *J Bacteriol* **186**: 7161–7174.
- Williams R., Peisajovich S. G., Miller O. J., Magdassi S., Tawfik D. S., and Griffiths A. D. (2006) Amplification of complex gene libraries by emulsion PCR. *Nat Methods* **3**: 545–550.
- Zerbino, D. R., and Birney, E. (2008) Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res* **18**: 821–829.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

- Appendix S1: Supplementary Material
- Appendix S2: Supplementary Material
- Appendix S3: Supplementary Material