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MiniReview

Catabolic mobile genetic elements and their potential use in bioaugmentation of polluted soils and waters

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Abstract

Genes that encode the degradation of both naturally occurring and xenobiotic organic compounds are often located on plasmids, transposons or other mobile and/or integrative elements. The list of published reports of such mobile genetic elements (MGEs) keeps growing as researchers continue to isolate and characterize new degrading bacteria and their corresponding degradative genes. There is also growing evidence that horizontal exchange of catabolic (degradative) genes among bacteria in microbial communities plays an important role in the evolution of catabolic pathways. Around 10 years ago the hypothesis was raised that we might be able to accelerate this natural gene exchange and pathway construction by introducing and subsequently spreading degradative genes, located on MGEs, into well established, competitive indigenous microbial populations as a means of bioaugmentation of polluted soils and waters. During the last decade, only a few reports on successful MGE- mediated bioaugmentation have been published. After summarizing the diversity of degradative MGEs, this review presents an overview of studies that have monitored the transfer of degradative genes in soil microcosms and in activated sludge and other wastewater treatment reactors, with emphasis on those that have clearly shown a direct effect of gene transfer on accelerated biodegradation. A few successful cases suggest that the strategy could indeed work under specific conditions, such as when the in situ degradation potential is absent and the pollutant degrading transconjugants can grow and become numerically dominant populations in the bacterial community. Further studies in this area are obviously needed to improve our current knowledge on the efficiency of gene dissemination as a tool in bioremediation.

Keywords: Bioaugmentation; Biodegradation; Catabolic plasmid; Catabolic transposable element; Soil; Wastewater treatment

1. Introduction

The role of mobile genetic elements (MGEs) and their horizontal transfer in evolution of bacterial genomes and adaptation of microbial populations to specific environmental changes is generally accepted nowadays. The most well-known example is the emergence and spread of antibiotic resistance genes as a result of the wide use of antibiotics [1]. Another example, which has been studied less extensively but is lately receiving increasing attention, is the adaptation of bacteria to environments contaminated with toxic xenobiotic (i.e., man-made) compounds [2]. During the last century industrial and agricultural activities have resulted in the release of large quantities of such compounds into diverse ecosystems. These chemicals create a strong selective pressure for the development of bacterial strains with novel or improved biodegradative capabilities. Indeed, under favorable environmental conditions indigenous microbial populations have evolved that show a remarkable ability to degrade a range of substrates previously considered to be recalcitrant. The genetic information encoding these metabolic activities is often found on plasmids or other mobile elements [2–4]. Detailed characterization of these different catabolic mobile elements sheds more light on their role in the in situ

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construction of new degradation pathways in bacterial communities. In addition, inoculation of these elements in polluted soils and waters and their subsequent transfer to indigenous populations may also be a feasible bioaugmentation approach. This review will first summarize the large diversity of degradative MGEs with emphasis on man-made compounds, and present evidence that they play an important role in the natural construction of degradative pathways. We will then further show how the transferability of these catabolic genetic elements may be used to accelerate biodegradation in soils and wastewater treatment reactors.

2. Catabolic MGEs and their role in bacterial adaptation to organic contaminants

MGEs that encode catabolic genes are considered to play a major role in the adaptation of microbial populations to xenobiotic organic compounds, which have been introduced in the environment during the past century. This occurs either by spreading the genes in a community and thereby increasing the diversity of organisms able to degrade these compounds, or by rearranging and combining pre-existing genes or gene fragments from different microorganisms to constitute a new pathway dealing with a new compound. Such catabolic mobile elements include plasmids, transposable elements and elements using phage-like integrases [2–4]. Interestingly, both on plasmids and in the chromosome, catabolic genes are often bordered by IS-elements (insertion sequences). These IS-elements may have played a role in recruitment of these genes by the replicon but also increase the potential of further exchange of the genes between different replicons and different hosts [5,6]. Typical examples of the different types of catabolic MGEs are listed in Table 1, with special emphasis on elements involved in xenobiotic compounds.

2.1. Catabolic plasmids

The first plasmids involved in degradation of organic xenobiotics were described ca. 20 years ago, and the list is increasing continuously [2]. Such plasmids have mostly been found in xenobiotic degrading bacteria after selective isolation of the strain. More recently also direct exogenous plasmid isolation has yielded several novel catabolic plasmids. This method consists of a mating, on plate or in situ, between a recipient strain and the total microbial community of a contaminated environmental sample followed by selection for recipient colonies that have acquired the expected degradative capacity. The catabolic plasmids are then recovered from those transconjugants and characterized. This has been successful for plasmids encoding degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) [7] and

Table 1
Overview of typical examples of the different types of catabolic mobile elements with emphasis on xenobiotic compounds

Mobile element	Strain	Substrate(s)	Size of MGE (kb)	Inc group	Reference
Plasmid					
pSS60	*Achromobacter* sp. LBS1C1	4-chlorobenzoate	53	P1	[48]
pBRC60	*Alcaligenes* sp. BR60	3CBA	75	P1	[5]
pAC25	*P. putida* AC858	3CBA	117	–	[49]
pENH91	*Alcaligenes eutropha* NH9	3CBA	78	P1	[50]
pJP4	*R. eutropha* JMP134	2,4-D	75	P1	[28]
pTSA	*Comamonas testosteroni* T-2	*p*-toluenesulfonic acid	85	P1	[51]
pPC170	*Pseudomonas cichori* 170	1,3-dichloropropene	60	P1	[52]
pCS1	*Pseudomonas diminuta*	parathion	68	–	[53]
pUU204	*Pseudomonas* sp. E4	2-chloropropionic acid	293	–	[54]
pC1-3	*Delftia acidovorans* CA28	3-chloroaniline	–	P1	[55]
pPS12-1	*Burkholderia* sp. PS12	1,2,4,5-tetrachlorobenzene	85	P1	[10]
Class I transposons					
Tn*5542*	*P. putida* ML2 (pHMT112)	benzene	12		[56]
Tn*5280*	*Pseudomonas* sp. P51 (pP51)	CB	8.5		[13]
Tn*5271*	*Alcaligenes* sp. BR60 (pBRC60)	chlorobenzoate	17		[12]
Tn*5707*	*R. eutropha* NH9 (pENH91)	3CBA	15		[56]
Class II transposons					
Tn*4651*	*P. putida* mt-2 (pWWO)	toluene, xylene	56		[4]
Tn*4653*	*P. putida* mt-2 (pWWO)	toluene, xylene	70		[4]
Tn*4656*	*P. putida* MT53 (pWW53)	toluene, xylene	39		[4]
Tn*4655*	*P. putida* G7 (NAH7)	naphthalene	38		[4]
Other elements					
Tn*4371*	*R. oxalatica* A5	biphenyl/4-chlorobiphenyl	55		[15]
bph-sal element	*P. putida* KF715	biphenyl/salicylate	90		[16]
bph-element	*P. putida* JHR	biphenyl	–		Springael, D., Dengis, P., Havel, J. and Reineke, W., unpublished results
clc element	*Pseudomonas* sp. B13	chlorocatechol	105		[3]

naphthalene [8]. Theoretically this method should allow to recover plasmids from both culturable and non-culturable bacteria, since cultivation of the original plasmid host is not required. It is however unknown until now if plasmids from non-culturable bacterial hosts have indeed been retrieved by using this method. One example of recruitment of indigenous catabolic genes in situ was reported by Focht et al. [9]. They observed in situ acquisition of biphenyl degradation genes in soil by a chlorobenzoate degrading inoculant, yielding a polychlorinated biphenyl (PCB) degrader. Although the nature of the elements involved in gene exchange in that study was not examined, this gene recruitment approach could be used to isolate various catabolic mobile elements from environmental samples without prior enrichment step.

Most of the catabolic plasmids reported up to now are rather large plasmids of more than 50 kbp and, if classified, belong to known incompatibility groups (Table 1). There seems to be an interesting distinction between plasmids that encode degradation of naturally occurring compounds and those that encode degradation of xenobiotics. Degradation of naturally occurring compounds seems to be often encoded by IncP-2 and IncP-9 plasmids (incompatibility grouping determined using fluorescent pseudomonads as hosts). On the other hand, degradation of compounds that are mostly man-made, such as several chloroaromatics, seems to be often encoded by the well-known broad host range (BHR) IncP-1 plasmids [2]. These plasmids are the most promiscuous self-transmissible plasmids characterized to date, with a host range that is much wider than that of IncP-2 and IncP-9 plasmids. IncP-1 plasmids were initially identified as bearers of antibiotic resistance genes, but it is now obvious that their promiscuity also plays an important role in the evolution of new metabolic pathways by recruiting catabolic genes or gene segments from different organisms into a suitable host [5,10]. Some catabolic plasmids that encode degradation of xenobiotics, including IncP-1 plasmids, clearly show indications of recent evolution of the determinants. For example, in the chlorobenzene (CB) degradative plasmids pP51 and pPS12-1 (IncP-1) (Table 1), genes for toluene degradation, allowing conversion of CB into chlorocatechols, have been clearly combined with genes encoding chlorocatechol degradation, thus forming a new pathway [10,11].

2.2. Transposons and other MGEs involved in pollutant degradation

Transposons that carry catabolic genes belong to both class I transposons and class II transposons (for a review see [4]). Class I consists of composite transposons in which the catabolic genes are flanked by two copies of very similar if not identical IS elements in direct or inverted orientation. Class II elements carry short terminal inverted repeats and transpose by the replicative mode in which transposases and resolvase are involved. Catabolic transposons are often located on plasmids. Well-known examples are the TOL and NAH7 class II transposons, and a few catabolic class I transposons that are carried by IncP-1 plasmids such as pBRC60 (Table 1).

A new type of catabolic mobile element has been recently identified in the PCB degrading bacterium *Ralstonia oxalatica* A5 (Table 1), which contains a 55-kbp biphenyl catabolic transposon Tn*4371* that allows the strain to completely degrade 4-chlorobiphenyl. In contrast with class I and class II transposons, Tn*4371* transposes by using an excision/integration mode rather than a replication mode. The transposon has been recently characterized at the molecular level and seems to have similarities to conjugative transposons [14,15]. It shows unusual combinations of phage-like integrase genes, *Pseudomonas*-like catabolic genes and plasmid-like transfer genes. Recently, other similarly organized mobile elements that encode biphenyl degradation were identified in other PCB degrading bacteria [14].

Another catabolic mobile element with bacteriophage-related functions is the so-called *clc*-element encoding degradation of chlorocatechols in the 3-chlorobenzoate (3CBA) utilizing *Pseudomonas* sp. B13 (Table 1). This 105-kbp element seems to use a bacteriophage-type integrase belonging to the phage P4 subfamily that promotes excision and subsequent integration into a new target DNA. Moreover, the element appears to integrate site-specifically into the glycine tRNA gene. Similar P4-type integrases have been found in various other mobile elements such as pathogenicity islands and symbiosis islands, but the *clc*-element integrase is about 250 amino acids longer than most other P4-type integrases. The *clc* element might be the representative of a new group of so-called 'catabolic gene islands' as other genes encoding degradation of other aromatics have been shown to be bordered by related integrase genes (for a review see [3]). For example in the case of the PCB catabolic genes of *Pseudomonas putida* JHR, exchange involving excision and subsequent integration in the new hosts chromosome of the corresponding catabolic operon has been shown (Springael, D., Dengis, P., Havel, J. and Reineke, W., unpublished data). In addition, other self-mobilizable chromosomal catabolic genes involving a discrete DNA segment have been described, but in these cases the element was not characterized at the molecular level [16].

In conclusion, genes that encode a wide range of catabolic enzymes involved in degradation of both naturally occurring and man-made compounds are carried by a wide diversity of mobile elements. There are several indications that these elements contribute to the natural spread of catabolic determinants in the environment using different transfer and integration or replication mechanisms. There is little direct information available however on where and when bacterial adaptation through catabolic gene exchange takes place in natural environments. One example

is a study on the natural horizontal transfer of naphthalene dioxygenase genes in a coal tar-contaminated field site. Strong evidence is provided that horizontal gene transfer had indeed occurred between members of the soil bacterial community, and that a naphthalene-catabolic plasmid may have played a role in adaptation of this community to the coal tar contamination [8]. A thorough investigation about the origin of CB degrading bacteria, which were isolated from CB-contaminated groundwater, and not from the uncontaminated region outside this area, shows strong evidence that bacterial adaptation to the pollution occurred due to genetic recombination among bacteria in the aquifer, leading to the formation of a novel pathway for CB degradation. This in situ genetic recombination apparently resulted in enhanced removal of CBs from that environment [17].

3. Applications of catabolic MGEs: an overview of bioaugmentation strategies involving inoculation and dissemination of degradative genes in soils and bioreactors

Bioaugmentation involves the inoculation of indigenous or allochtonous wild-type or genetically modified organisms into polluted soils or bioreactors in order to accelerate the removal of undesired compounds. It may be a suitable strategy in cases where spontaneous biological degradation of aromatic pollutants in the environment proceeds slowly due to insufficient degradation potential and/or slow adaptation of the indigenous microorganisms. This augmentation of natural bacterial populations with highly efficient laboratory strains is an attractive approach, and has been shown in several studies to have a positive effect on the degradation of different organic compounds in soils [18] and waste(water) treating bioreactors [19]. Maintaining high levels of these active inoculants in these systems is however a great challenge, as these strains have to compete with indigenous microorganisms for resources and space, face predation by e.g. protozoa and are exposed to unfavorable conditions. Both good and poor survival and activity of inoculants has been observed in soils [20,21], activated sludge, and anaerobic reactors [22–25].

To avoid the need for the inoculum to survive and remain functional for prolonged periods, an alternative strategy has been suggested and examined by a few groups. It focuses on the inoculation of strains with MGEs that encode the relevant genetic information and their subsequent horizontal transfer to one or various well-established and competitive indigenous bacterial populations of an ecosystem. In this case, the survival of the introduced donor strain is no longer needed once the catabolic genes are transferred and expressed in the indigenous bacteria. The first papers in which this strategy was suggested for water or soil remediation were only published ca. 10 years ago [26,27], and were based on the findings that degradative pathways were often located on plasmids that are self-transferable to different species and even different genera in plate matings [28]. Moreover, transfer of conjugative plasmids at detectable frequencies in soils and bioreactors had been demonstrated under simulated natural conditions (for recent reviews on gene transfer in soil, see [29,30]). Up to 10–12 years ago, most of those studies had however been performed with plasmids that encode antibiotic or heavy metal resistance, and not degradation of organic contaminants. The rest of this paper will briefly review the studies that have investigated the effect of inoculation and subsequent transfer of catabolic genes on in situ biodegradation in soils and bioreactors.

3.1. Examples of plasmid-mediated bioaugmentation of soils

The effect of transfer of a degradative plasmid in soil on the degradation of the corresponding organic contaminant has only been examined for a few catabolic plasmids. In 1991, Brokamp and Schmidt [26] were the first to suggest the possible role of catabolic plasmid transfer in enhanced pesticide degradation in soil. They could however not present direct evidence for transfer-mediated biodegradation of the pesticide 2,2-dichloropropionate, since the inoculum itself survived and could degrade the herbicide.

The first report of a positive effect of the transfer of *bph* genes from an inoculated donor strain to soil bacteria on biphenyl degradation in soil was given by de Rore et al. [31]. The donor, *Enterobacter agglomerans* DK3 harbored plasmid RP4::Tn*4371*, an IncP-1α plasmid in which Tn*4371* (see Section 2.2 and Table 1), encoding biphenyl degradation, has been inserted. In spite of the fast disappearance of the donor strain, which was unable to use biphenyl as carbon and energy source, the plasmid was transferred in soil to different bacteria, which expressed the *bph* genes. Under most of the conditions tested, including nutrient-amended and non-amended soil, the amount of biphenyl removed and oxygen consumed during a 3–5-week period was higher in the inoculated soil than in the non-inoculated soil. The detection of transconjugants together with the poor survival of the donor and its inability to degrade biphenyl indicated that the enhanced degradation of biphenyl was due to transconjugants that had acquired and expressed the *bph* genes. Very recently, transfer of the same plasmid RP4::Tn*4371* was compared in moist and desiccating soil [32]. The transconjugants that were isolated from the desiccated soil were much more drought resistant than those from the moist soil. This new concept of selecting drought resistant bacteria to use for bioaugmentation presents an interesting avenue, since tolerance to desiccation is needed for inocula in order to have a long shelf life with full maintenance of activity in a desiccated form, and to survive drought periods in soil.

During the last 6 years several studies have shown that

transfer of 2,4-D degradative plasmids to soil bacteria can have a positive effect on the degradation of 2,4-D, ranging from small and not significant to very pronounced. diGiovanni et al. [33] demonstrated transfer of the IncP-1β plasmid pJP4 from *Ralstonia eutropha* JMP134 to indigenous soil bacteria when very high concentrations of 2,4-D (1000 mg kg^{-1}) were added to the soil, but not when 500 mg 2,4-D kg^{-1} was spiked. Since this formation of transconjugants in the presence of 1000 mg kg^{-1} 2,4-D was accompanied by accelerated degradation, and the donor died off rapidly in this soil the transconjugants were thought to be responsible for the enhanced degradation.

To be able to distinguish degradation by the donor from degradation by transconjugants, Newby et al. [34] performed similar experiments as those of diGiovanni et al. [33] using *Escherichia coli* as a donor strain, which is unable to degrade 2,4-D even when it contains plasmid pJP4. They showed transfer of pJP4 to different indigenous bacteria in some but not all of the tested soils, and observed a significantly higher number of transconjugants in 2,4-D (500 or 1000 mg kg^{-1}) treated than in non-treated soils. The 2,4-D degrading transconjugants belonged to the genera *Pseudomonas* and *Burkholderia*. The positive effect of this plasmid transfer on accelerated 2,4-D degradation was very small and only shown for one of the four soils studied and in the presence of 1000 mg kg^{-1}. The same group [35] extended their work by repeating their pJP4 transfer experiments in pilot-scale soil bioreactors spiked with 500 mg kg^{-1} 2,4-D, and using both *E. coli* and *R. eutropha* JMP134 as donors. In the reactors there was no evidence for gene transfer-mediated degradation. However, when soil samples taken from the reactors after 49 days were incubated in microcosms and spiked again with 500 mg kg^{-1} 2,4-D, increased degradation was now observed in the soils that had been previously inoculated with the *E. coli* donor. Together with the detection of large numbers of transconjugants (belonging to the genera *Burkholderia* and *Ralstonia*), these data indicate that the spread of pJP4 led to the development of transconjugants that were unable to significantly decrease the 2,4-D concentration in the bioreactor during the first 50 days, but under microcosm conditions they could rapidly degrade a new dose of 2,4-D, added 50 days later. A plausible explanation for their poor performance in the soil reactor could be their low initial numbers. Alternatively, the conditions in the microcosms may have been different enough from those in the reactor to stimulate the growth and/or activity of the transconjugants. Further work by this group was carried out in columns with 2,4-D-contaminated soil (again 500 mg kg^{-1}) under unsaturated or saturated flow conditions. One week after inoculation on the top of the columns run under unsaturated conditions, pJP4 was detected in both donor and transconjugant cells within the upper 10.5 cm. In saturated soil, no transconjugants were detected but donors were found throughout the entire length of the column (30.5 cm). The lack of detectable gene transfer here could be either due to lack of cell contact in a water-saturated soil, or due to the anoxic conditions, which prevented transconjugants to grow and become detectable. The observed gene transfer in non-saturated columns resulted only in a small improvement in 2,4-D removal compared to the uninoculated control (ca. 10% of the total 2,4-D added) [36]. One major pitfall in these four studies on pJP4-mediated bioaugmentation is the very high concentrations of 2,4-D used, ranging from 500 to 1000 mg kg^{-1} soil. Taken together the studies suggest that transfer of pJP4 to indigenous soil bacteria improves the 2,4-D degradation rate only in some cases, and even then only moderately.

In parallel, we [37–39] examined the effect of conjugative transfer of a few different 2,4-D degradative plasmids on 2,4-D degradation in soil. This was done at 2,4-D concentrations that were much lower (100 mg kg^{-1}) than in the studies described above. First, transfer of two 2,4-D degradative plasmids, pEMT1 and pEMT3 [7], from an *E. coli* donor to the indigenous soil bacteria was monitored in soil microcosms, and the presence of 2,4-D had a clearly positive effect on the numbers of transconjugant formed (Fig. 1a,b) [37]. A few 2,4-D degrading transconjugants containing pEMT1 were identified by FAME analysis as species belonging to *Stenotrophomonas* and *Ralstonia*. Enhanced degradation of 2,4-D was clearly seen in one of the two soils tested (Fig. 1c), and since the *E. coli* donor strain was unable to degrade 2,4-D, the improved 2,4-D degradation in soil was clearly due to plasmid transfer. Since *E. coli* is not a suitable strain for bioremediation purposes, the previous study was extended with the same soil, now using *P. putida* UWC3 as donor of plasmid pEMT1. Again, transfer of plasmid pEMT1 was observed and correlated with an enhanced degradation of 2,4-D. A few transconjugants that harbored pEMT1 and degraded 2,4-D were identified as *R. eutropha* [38]. Since the donor strain was still present when degradation started and was recently shown to partially degrade 2,4-D in liquid cultures and sterile soil [39], its involvement in the degradation of 2,4-D could not be totally excluded. In a following study, we compared the bioaugmentation potential of inoculation and transfer of the 2,4-D degradative plasmids pEMT1 and pJP4 using the same *P. putida* donor strain in the A (0–30 cm)- and B- (30–60 cm) horizon of a sandy-loam soil [39]. The two soils have a different texture, organic matter content and thus also a different microbial community. Interestingly, bioaugmentation with both plasmids was most successful in the B-horizon soil, where the indigenous microbial community could not degrade the herbicide (100 mg kg^{-1}), even up to 3 months, while inoculation of the donor and subsequent plasmid transfer resulted in complete removal of 2,4-D within 19 days. In the case of plasmid pEMT1, this success must be due to plasmid transfer to the indigenous bacteria since the donor died off very rapidly. In addition, DGGE analysis (denaturing gradient gel electrophoresis) of the soil 16S rRNA

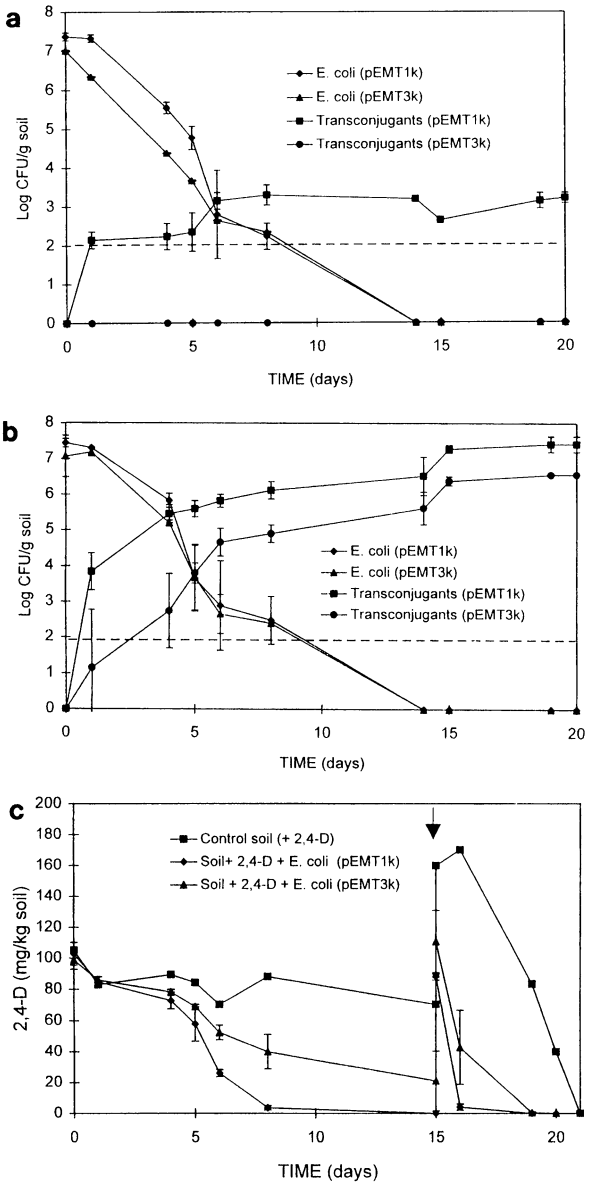

Fig. 1. Survival of the donor strain *E. coli* Xl1-Blue (pEMT1) and Xl1-Blue (pEMT3) and development of transconjugants in untreated (a) and 2,4-D-treated (b) soil and disappearance of 2,4-D (c). The dotted line represents the detection limit; when no error bars are visible, they are hidden behind the symbols (after Top et al. [37]). (With kind permission of Kluwer Academic Press.)

gene pool of the B-horizon soil revealed the in situ formation and subsequent proliferation of high numbers of 2,4-D degrading transconjugants. This confirms that the inoculation and spread of a catabolic plasmid enables specific indigenous bacterial species to acquire new useful catabolic genes, which allows them to degrade the contaminant and to become numerically dominant members of the community. Taxonomical analysis of 95 2,4-D utilizing transconjugants from this study has shown that the vast majority belonged to the genera *Burkholderia* (four species) and *Ralstonia* (two species), and very few to *Stenotrophomonas maltophilia*. There was no major difference in host range between pJP4 and pEMT1, and the transconjugants found in the A- and B-horizons belonged mainly to the same species [57]. This is the first study that shows such a drastic increase in degradation potential of a soil (B-horizon) due to transfer of inoculated catabolic genes to indigenous bacteria.

It is clear from these different studies on 2,4-D degradation, independently performed in two different laboratories, with soils from different continents, and with different donor strains and two different 2,4-D degradation plasmids, that these catabolic plasmids were most often transferred and expressed in strains that belong to the genera *Burkholderia*, *Ralstonia* and *Pseudomonas*. The finding that the β-subgroup Proteobacteria (including the first two genera mentioned above) seem to be the best recipients for these plasmids corresponds very well with the results obtained in a study of Fulthorpe et al. [40]. They found that in a collection of 32 2,4-D degrading degrading bacteria isolated from different locations in the world, 2,4-D degradative genes identical or similar (60–100% sequence similarity) to those of plasmid pJP4 were mainly found in strains that belong to the β-subgroup Proteobacteria. The limited spectrum of hosts to which these so-called broad host range plasmids, such as the IncP-1β plasmids pJP4 and pEMT3, and the non-IncP-1 plasmid pEMT1, transferred in soil is clearly not due to limited transfer abilities of these plasmids. They have been found to transfer to a much wider range of genera when transconjugants are selected for resistance to Hg or an antibiotic instead of growth with 2,4-D as sole carbon source [7,28]. The limitation to their spread and maintenance can most probably be explained by the inability of several other species to efficiently express the catabolic genes. Thus in these microcosm experiments described above, the plasmids may have transferred to various other bacterial groups, but those transconjugants did not proliferate or even lost the plasmid again since the catabolic genes were not expressed in these hosts and thus did not confer a selective advantage.

Inoculation and subsequent transfer of catabolic genes as a strategy to accelerate biodegradation of undesirable organic compounds in soil has also been suggested in the context of rhizoremediation. Crowley et al. [41] observed increased 2,5-dichlorobenzoate (2,5-DCB) degradation in soil that was planted with *Phaseolus vulgaris* and inoculated with a *Pseudomonas fluorescens* strain that contained a plasmid encoded 2,5-DCB degradation pathway. Since 2,5-DCB degrading bacteria different from the inoculum were only found in inoculated soils, these bacteria were thought to be transconjugants that had received the catabolic plasmid from the donor. The overall enhanced degradation of 2,5-DCB in the inoculated and planted soil may have been partially due to this plasmid transfer, but hard evidence was lacking. Sarand et al. [42] inoculated a *P. fluorescens* soil isolate, provided with the TOL plasmid, in soil microcosms with and without pine seedlings, my-

corrhized with *Suillus bovinus*. After 3 months of regular treatment with *m*-toluate (mTA) the catabolic plasmid was found to be transferred into indigenous bacteria. The removal of mTA from this soil was not measured analytically, but the inoculation seemed to protect the plant and fungus from mTA. Since the donor could not be detected anymore, this protective effect was attributed to the degradation of mTA by the TOL plasmid containing indigenous transconjugants. The rhizosphere may be a habitat that allows higher frequency of catabolic gene transfer as well as higher metabolic activity compared to bulk soils, both of which are necessary for a successful plasmid-mediated bioaugmentation approach. Rhizoremediation by means of catabolic gene transfer may thus be a very valuable approach that deserves further investigation.

Most of the studies summarized above show first of all that a selective pressure, here an organic compound that can be degraded by plasmid encoded enzymes, can strongly affect the extent of gene spread in a soil habitat, even when the donor dies off quickly. This positive effect of the presence of the pollutant on the number of transconjugants is almost certainly due to the proliferation of the transconjugants, and not to a direct effect of the pollutant on the conjugation efficiency. Accelerated degradation of the pollutants after transfer of the introduced plasmid to indigenous soil bacteria and subsequent growth of these transconjugants was obtained in a few cases, and was often clearly attributed to the activity of in situ formed transconjugants. Due to the large amount of variables in these different studies (soils, contaminants, donor strains, plasmids, inoculation and incubation procedures, etc.) it is very difficult to conclude which factors determine the success of such a bioaugmentation strategy, but a few points can be raised. It is clear that the transconjugants have to reach high population sizes to significantly affect the degradation rate in soil. In successful cases, the transconjugant numbers were often ca. 10^6 CFU g^{-1} soil or higher (often 1–100% of the total heterotrophic plate counts of the soils). As discussed above, the conjugation event itself is unlikely to produce transconjugant numbers of 10^6 CFU g^{-1} soil or higher. Often low numbers (10^1–10^4 CFU g^{-1}) are observed the first days after inoculation, which rapidly increase within the first week (Fig. 1). In order to grow to such high densities, the transconjugants need to have a selective advantage over the other indigenous bacteria. In the case of high contaminant concentrations such as in all studies described here (at least 100 mg kg^{-1} soil for 2,4-D), the contaminants seemed to support selective growth of the transconjugants and the bioaugmentation strategy worked in several cases. There is however no evidence that lower more realistic concentrations of the pollutants would also allow the formed transconjugants to increase their population size. Low pollutant concentrations may not be able to support growth of transconjugants, and the cells would need another selective advantage to successfully compete with the indigenous bacteria for resources and space. More sensitive and accurate methods for monitoring low densities of transconjugants and low pollutant concentrations in soil will help to answer these questions in the future. The rather low recalcitrance of the model compounds used so far in these studies is an additional weak point. Although the period for complete degradation was longer in most non-inoculated soils than after inoculation, the indigenous bacteria eventually degraded the compounds, which makes a bioaugmentation project seem superfluous. From the viewpoint of soil bioremediation it would therefore be more relevant to understand how more recalcitrant pollutants (which accumulate in the environment) can be rapidly removed by spreading the corresponding mobile catabolic genes. The few cases described above, where the indigenous catabolic potential was very low and catabolic gene transfer decreased the degradation time with several months [39] or protected biological life in the soil [42], show however that this plasmid-mediated bioaugmentation could be a feasible strategy under such conditions.

3.2. *Catabolic plasmid transfer and its potential in the bioaugmentation of activated sludge and other wastewater treating bioreactors*

Very few studies have evaluated the introduction of degradative bacteria with mobile catabolic genes into activated sludge or different wastewater treatment reactor systems in order to enhance biodegradation of recalcitrant organic compounds. In most of these studies the exact role of the indigenous transconjugants in accelerated degradation was never investigated in detail. An overview of the studies that have been performed in this area is presented below.

In 1989 McClure et al. [43] monitored the survival of a *P. putida* donor strain that harbored a 3CBA degradative non-conjugative IncQ plasmid in a lab-scale activated sludge unit that received 3CBA, and also monitored the potential transfer of the plasmid to the indigenous bacteria as well as the degradation of 3CBA. Despite the long survival of the inoculum in the sludge no 3CBA removal was observed. Although transfer of conjugative plasmids from sludge bacteria to the inoculant was observed, as well as mobilization of the catabolic plasmid to sludge bacteria in separate plate matings, the data did not support the hypothesis that spreading catabolic genes directly into an activated sludge community by plasmid mobilization could accelerate pollutant degradation.

Nüsslein et al. [24] introduced a genetically modified *Pseudomonas* sp. strain B13FR1(pFRC20P) (designated FR120), which contains the non-conjugative but mobilizable plasmid pFRC20P, and simultaneously degrades 3CBA and 4-methylbenzoate (4MBA). Three days after inoculation of strain FR120 a drastic decrease in the concentrations of 3CBA and 4MBA (initially 1 mM each) was observed in comparison with a non-inoculated reactor.

Since plasmid pFRC20C cannot initiate its conjugative transfer due to lack of transfer genes, its spread to sludge bacteria was detected infrequently in the activated sludge, due to the presence of mobilizing plasmids in the sludge. Conclusions on the effect of this low mobilization frequency on the rate of biodegradation of the pollutants could however not be drawn.

The transfer of the mobile *clc* element, which codes for chlorocatechol degradation (see Section 2.2) to bacteria in activated sludge and membrane reactors has been demonstrated in three different studies. First *Pseudomonas* sp. strain B13 was used as donor and *P. putida* F1 was co-inoculated as possible recipient strain [44]. When strain F1 receives the *clc* element, it is able to degrade CBs by complementation of two partial degradative pathways, while both the donor strain B13 and the recipient strain F1 cannot degrade these compounds. Transfer of the *clc* element to the inoculated recipient in an activated sludge microcosm was only detected when cell numbers of strain B13 were sufficiently high thanks to the addition of 3CBA (a substrate for the donor) and 1,4-DCB. The effect of this gene exchange on the degradation of this pollutant was however not investigated.

Indications that spread of the *clc* element in activated sludge increased the degradation of shockloads of 3CBA was recently presented by Springael and coworkers ([45]; Springael, D., Ghyoot, W., Nuyts, S., Van Roy, S. and Diels, L., unpublished data). They inoculated *P. putida* BN210, which carries the *clc* element on the chromosome, in two different activated sludge systems, i.e., a conventional activated sludge system (CAS) and an activated sludge system coupled to a membrane separator device (CAS–MS), which increases the biomass density in the main reactor unit. In the CAS–MS system, the community started to degrade 3CBA more rapidly than in the CAS reactor, while in both reactor systems the donor strain BN210 disappeared. Moreover, in the CAS–MS reactor, a 3CBA degrading population developed more rapidly than in the CAS reactor. By using PCR–DGGE analysis of the *clc* genes, it was shown that the *clc* element remained present at high levels in the reactor in spite of the declining donor population, which suggests that this element had transferred to indigenous bacteria.

In situ horizontal transfer of the *clc* element from the same *P. putida* strain was also demonstrated in a membrane biofilm reactor that treated model wastewater containing 3CBA [25]. Cells that grow in a biofilm conformation are in very close contact, which may facilitate the transfer of MGEs. The inoculated strain became undetectable in the reactor and was replaced by several novel 3CBA-mineralizing populations that were responsible for removal of 3CBA from the water. In the non-inoculated reactors, 3CBA was not degraded and 3CBA degraders could not be isolated. Southern blot hybridization of genomic DNA using *clc*-element-specific probes and field inversion gel electrophoresis analysis indicated the presence of the complete *clc* element in one or more copies in the isolates, which could transfer the *clc* genes to recipient strains. These data suggest that the novel 3CBA degrading populations were formed by in situ horizontal transfer of the *clc* element from the inoculum to indigenous bacteria, which were more competitive under the defined reactor conditions than the inoculated strain.

The above summary of very limited successes of bioaugmentation of wastewater treatment reactors by means of catabolic gene transfer suggests that this approach seems less feasible in these systems than in soils. However, there are also a lot less studies published on gene transfer in these reactors and very little is known about the conditions that promote or inhibit exchange of MGEs. A better understanding of conjugative gene exchange in these wastewater treatment systems could thus help to improve the success of bioaugmentation by plasmid transfer [19]. One of the main conditions for conjugative plasmid transfer is cell density, since donor and recipient strains must be in close contact. Mancini et al. [46] examined plasmid transfer in an activated sludge unit and found the highest numbers of transconjugants on the bottom of the primary clarifier and in the return-activated sludge from the secondary clarifier, where cell densities are the highest. The positive effect of surfaces to protect the pili has also been reported. Especially BHR plasmids encode short rigid pili that may break more easily and are therefore stabilized by solid surfaces [47]. Because of their high cell density and the presence of biodegradable organic matter, sludge flocs, which contain the majority of the bacteria present in activated sludge, may form an ideal site for conjugation. If this is true, successful integration of donors and subsequent plasmid transfer in activated sludge could be enhanced by using donor cells that easily form aggregates. Because of the porous structure of flocs it is however not clear whether the bacteria really have a greater chance to meet. More research is needed to understand which are the hot spots for conjugative gene transfer in activated sludge. In spite of the limited amount of data on bioaugmentation by catabolic gene transfer in activated sludge and other wastewater treatment reactors, there is no reason to believe that the catabolic potential of these ecosystems could not be augmented by introducing and disseminating catabolic MGEs.

4. Conclusions

In conclusion, this review shows that the cases of clear correlations between observed transfer of catabolic genes in soil or wastewater treatment systems and clearly improved biodegradation of an organic pollutant are still very limited. A few successes indicate that the approach of plasmid-mediated bioaugmentation can be feasible in cases where the limiting factor for biodegradation in soil and in wastewater treatment reactors is the absence of the

necessary catabolic potential in the indigenous communities. The difficulty of finding a suitable combination of a recalcitrant pollutant, a corresponding degradative mobile element, a donor strain that can be counterselected and that either dies off over time or does not degrade the compound, and sensitive methods for detecting gene transfer and low pollutant concentrations, explains in part why the reported cases of successful plasmid-mediated bioaugmentation are still so limited. Furthermore, it is clear that in spite of the many studies on gene transfer in soil, and the fewer reports in bioreactors, our basic understanding of factors that influence the dissemination and fate of introduced genes in such systems needs to be improved. This includes understanding the conditions that stimulate conjugation and other gene transfer mechanisms in these ecosystems, the natural host-range of several catabolic MGEs and the expression range of their catabolic genes, the competitive fitness of the transconjugants, and the maintenance of the MGEs both in the absence of the corresponding substrate and at concentrations that are much lower (ppb) than those applied so far in most bioaugmentation studies. The success of plasmid-mediated bioaugmentation in the future will largely depend on increased knowledge in these areas.

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