Plasmid-Mediated Tolerance Toward Environmental Pollutants

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ABSTRACT The survival capacity of microorganisms in a contaminated environment is limited by the concentration and/or toxicity of the pollutant. Through evolutionary processes, some bacteria have developed or acquired mechanisms to cope with the deleterious effects of toxic compounds, a phenomenon known as tolerance. Common mechanisms of tolerance include the extrusion of contaminants to the outer media and, when concentrations of pollutants are low, the degradation of the toxic compound. For both of these approaches, plasmids that encode genes for the degradation of contaminants such as toluene, naphthalene, phenol, nitrobenzene, and triazine or are involved in tolerance toward organic solvents and heavy metals, play an important role in the evolution and dissemination of these catabolic pathways and efflux pumps. Environmental plasmids are often conjugative and can transfer their genes between different strains; furthermore, many catabolic or efflux pump genes are often associated with transposable elements, making them one of the major players in bacterial evolution. In this review, we will briefly describe catabolic and tolerance plasmids and advances in the knowledge and biotechnological applications of these plasmids.

INTRODUCTION

Since the Industrial Revolution, there has been an increasing pace in the production of environmentally hazardous compounds that deliberately or accidentally have reached waters and soils, polluting them. The survival capacity of microorganisms in a contaminated environment is limited by the concentration and/or toxicity of the pollutant. Some contaminants are able to disrupt the normal development of the cell, others induce mutations, and some of these can kill cells at very low concentrations. Through evolutionary processes, some bacteria have developed or acquired mechanisms to cope with the deleterious effects of toxic compounds, permitting normal cellular subsistence in polluted environments—a phenomenon known as tolerance. Common mechanisms of tolerance include the extrusion of contaminants to the outer media and, when concentrations of pollutants are low, the degradation of the toxic compound. For both of these approaches, plasmids play an important role in the evolution and dissemination of these catabolic pathways and efflux pumps.

In this article, we will briefly describe catabolic and tolerance plasmids and advances in the knowledge and biotechnological applications of tolerance plasmids.

ENVIRONMENTAL CATABOLIC PLASMIDS

The mineralization of pollutants decreases their concentration in the environment and therefore allows better survival of the organism; at the same time, pollutants are used to obtain energy for growth. This mechanism of resistance is only useful when the contaminant concentration is moderate or low enough to allow normal bacterial metabolism; for example, while Pseudomonas putida mt-2 is able to mineralize toluene through the TOL pathway, the strain fails to thrive in high concentrations of toluene (1).
Environmental plasmids are often conjugative and can transfer their genes between different strains; furthermore, many catabolic genes are often associated with transposable elements, making them one of the major players in bacterial evolution. Genes for the degradation of contaminants such as toluene, xylenes, alkanes, naphthalene, phenol, nitrobenzene, triazine, and others have been reported to be encoded in plasmids (2, 3, 4). Catabolic plasmids are generally large in size (60 to 200 kb), making them difficult to isolate and complicating the ascription of genes to these extrachromosomal elements. Recent advances in sequencing techniques have significantly advanced our knowledge of genes that are present in noncultivatable bacteria; however, it has been suggested that the presence of plasmid-encoded genes in metagenomic libraries is often underrepresented (5), leaving a knowledge gap regarding the role and importance of plasmids harbored by noncultivatable bacteria.

It should be noted that the presence of catabolic genes has not only been identified in plasmids, but also in the chromosome. Here, we describe plasmid-based systems that have been studied in detail.

Plasmids That Encode Pathways for the Degradation of Monoaromatic Compounds

Toluene is a natural product and, although aerobic bacteria have evolved several different pathways to degrade it, they all share a common strategy. In many cases, an upper pathway is able to activate the aromatic ring throughout mono- or dioxygenases, or by oxidation of the alkylic substituent; subsequently, the lower pathway breaks down the activated aromatic ring to produce intermediates that can enter the tricarboxylic acid cycle (6, 7). The most studied plasmid for the degradation of monoaromatic compounds is the TOL plasmid (pWW0), which encodes the genes necessary for the degradation of toluene, m- and p-xylene. This plasmid was originally identified in P. putida mt-2 and has since been the subject of intensive research (8). pWW0, a 117-kbp self-transmissible plasmid belonging to the IncP-9 incompatibility group, was completely sequenced in 2002 (9). Together with toluene catabolic genes, the plasmid encodes all the functions necessary for its replication, stable inheritance, and conjugation. The catabolic genes within the pWW0 plasmid are organized in two operons, the “upper pathway” (xylUWCABN), which converts toluene and xylenes into benzoate and methyl benzoates, and the “meta pathway” (xylXYZLFEGFJQKIH), which transforms these intermediates into Krebs cycle intermediates (Fig. 1). Two regulatory proteins, XylR and XylS, control the expression of these two operons through a fine-tuned regulatory circuit that has also been studied in great detail (10, 11). In fact, this regulatory system represents a paradigm for signal integration in gene regulatory networks (12). These two operons, together with the 14 open reading frames between them, are located between two identical repeats of 1275 bp. This complete region has been named IS1246 because of its insertion sequence characteristics. Spontaneous deletion of a 39-kbp region comprising the catabolic genes has been observed, probably as a consequence of recombination between the two repeated sequences (13, 14). Two transposable elements, with characteristics of class II transposons, have been identified in the pWW0 plasmid (Tn4651 and Tn4653) (15, 16); and another insertion sequence (ISPpn12) is also present and active in this plasmid (17). The presence of multiple insertion sequences, transposases, and recombinases is a common element in most catabolic plasmids, and it is thought to be related to the acquisition of new genes and microbial evolution.

Plasmids that carry xyl genes homologous to those of the pWW0 plasmids are normally referred to as TOL plasmids and have been isolated from different locations (18, 19, 20, 21). They differ in the organization of the catabolic pathways, in size, and even in replicon type; e.g., plasmids pWW53 and pDK1 belong to the IncP-7 plasmid family (22, 23). Comparison between multiple TOL plasmids indicate that transposition-related genes and sites have contributed to the diversification of plasmid structures and to the dissemination of common gene clusters to various plasmids (22).

Plasmid pTOM (latter designated pBV1E04) of Burkholderia vietnamensis G4 (24; CP_000620) encodes the toluene 2-monoxygenase (T2MO) that catalyzes the two initial oxidation steps in toluene degradation, transforming toluene into 3-methylcatechol via o-cresol (25). Ring cleavage proceeds via a meta cleavage pathway that is also encoded in the pBV1E04 plasmid. Although it is known that T2MO activity is inducible, the regulation of this pathway has not been studied; however, a gene with homology to the XylR/NtrC family of transcriptional regulators that is located upstream of the T2MO gene cluster has been hypothesized to be the regulatory protein of the operon (6). B. vietnamensis G4 was initially isolated because it oxidized trichloroethylene (TCE), but it was later demonstrated that it can grow in the presence of toluene, phenol, o-, m-, and
p-cresol, and benzoate\(^{(26)}\). This strain and the\(^{p}\) TOM plasmid have been used extensively for different biotechnological approaches, including TCE and toluene rhizoremediation (see below). Interestingly, the backbone of the pBV1E04 plasmid is quite similar to that of pGRT1 from the solvent tolerant \(P.\ putida\) DOT-T1E strain \(^{(27)}\). Several transposases, insertion sequences, and integrases are encoded in the pBV1E04 plasmid, many of them surrounding the area where the catabolic genes are located, suggesting transfer of catabolic genes to/from other strains.

**Plasmids That Encode Pathways for the Degradation of Polycyclic Aromatic Hydrocarbons and Heteroaromatic Compounds**

Since the first report of a plasmid that encoded the catabolism of camphor, the CAM plasmid \(^{(28, 29)}\), various plasmids that confer the ability to grow in polycyclic aromatic compounds have been isolated. Naphthalene has served as a model compound for the study of polycyclic aromatic hydrocarbons (PAH) degradation and the associated plasmids have been named as NAH plasmids. Naphthalene degradation enzymes are encoded within two operons; the *upper pathway* encodes functions for the conversion of naphthalene to salicylate (Fig. 1), and the *lower pathway* encodes genes for the *meta*-ring fission pathway and a predicted methyl-accepting chemotaxis protein, NahY \(^{(30, 31, 32, 33, 34, 35)}\). The *nahR* gene encodes the regulatory protein of the system, which belongs to the LysR family \(^{(36)}\) and induces the two operons in the presence of salicylate.

Plasmids pDTG1 from \(P.\ putida\) strain NCIB 9816-4, pND6-1 from *Pseudomonas* sp. strain ND6, and NAH7 from \(P.\ putida\) G7 have been completely sequenced \(^{(37, 38, 39)}\). These three plasmids encode the *nahR* gene, as well as two catabolic operons: the *nah* operon, which encodes the *upper* pathway, and the *lower* operon for salicylate degradation (Fig. 1). Proteins for naphthalene degradation are almost identical in the three strains (99% to 100% identity in amino acid sequences), with the exception of two duplicated genes in pND6. Furthermore, nucleotide identity in the catabolic region of plasmids pND6 and pDTG1 is >99%, including a 15-kbp region between the two operons that contain a number of functionally unrelated genes \(^{(38)}\). Despite this homology, \(P.\ putida\) strain NCIB 9816-4 degrades naphthalene through a chromosomally encoded *orthogonal pathway* due to the presence of an ISP\(\text{Pre1}\) between the *nahG* (encoding the salicylate hydroxylase) and *nahT* genes of the pDTG1 plasmid \(^{(37)}\). In pND6 and pDTG1, the two operons are transcribed in opposite directions, while, in NAH7, both operons are transcribed in the same direction. *nahR* in NAH7 is not located upstream of the *lower* operon, but in between the two operons \(^{(38)}\). In this plasmid, the naphthalene degradation genes are located within a class II transposon (Tn\(4653\)), which lacks a *tnpA* gene, but that can be mobilized by the action of the TnpA from pWW0 Tn\(4653\) supplied in trans \(^{(40)}\). Within these three plasmids, numerous transposases, resolvases, and integrases are located in the vicinity of the catabolic genes, and, in many cases, they are similar to elements found in catabolic plasmids pWW0 and pCAR1. The “backbone” of the pDTG1 and NAH7 plasmids is homologous to that of the pWW0 plasmid, suggesting that the exchange of catabolic genes by means of horizontal gene transfer may have occurred between these plasmids.

Plasmids involved in the degradation of polycyclic aromatic hydrocarbons with three aromatic rings have also been described (e.g., the NAH-plasmid-encoded pathway is able to mineralize phenanthrene and anthracene \(^{(41)}\)). Not only pseudomonads, but many other bacteria are able to degrade naphthalene and phenanthrene through similar biochemical steps. The enzymes of the naphthalene pathway are also able to degrade phenanthrene and anthracene via a 1-hydroxy-2-naphthoic acid intermediate, which is subsequently oxidized through salicylate and catechol (in other cases, this intermediate can be channeled to \(o\)-phthalate and protocatechuate \(^{(42)}\)). The *Burkholderia* sp. strain RP007 contains a plasmid that encodes the upper pathway for the degradation of naphthalene and phenanthrene \(^{(43)}\). The *Sphingomonas aromaticivorans* F199 plasmid known as pNL1 encodes genes associated with the degradation of biphenyl, naphthalene, \(m\)-xylene, and \(p\)-cresol. Interestingly, although the related genes are organized in operons under a commonly occurring pseudomonad regulatory scheme, in sphingomonads the genes are scattered across the plasmids \(^{(44, 45)}\). In the pNL1 plasmid, genes associated with aromatic degradation are distributed among at least 11 transcriptional units (Fig. 1). The unusual coclustering of genes associated with different catabolic pathways (biphenyl, toluene, xylenes, and naphthalene) observed in this plasmid is likely due to evolutionary modifications applied to similar biochemical mechanisms for the degradation of intermediates in the different pathways \(^{(46)}\). As in many other catabolic plasmids, the presence of a recombinase, an excisionase, a phage-type integrase-recombinase, and two transposons have been identified.
The genus *Sphingomonas* is able to degrade a wide variety of xenobiotics (biphenyl, PAHs, and substituted PAHs, carbazole, diphenyl ethers, furans, dibenzo-p-dioxins, and others) and many of the catabolic genes for these compounds are located on plasmids that can be mobilized to other *Sphingomonas* strains (45, 47, 48). Plasmid pBN6 of *S. xenophaga* BN6 encodes the pathway involved in the degradation of naphthalene sulfonate, and the related genes show high similarity to those encoded in the pNL1 plasmid of *S. aromaticivorans* F199 and also share the structural organization of the naphthalene, biphenyl, and phenanthrene genes within three transcriptional units. Not surprisingly, these genes are flanked with genes that encode putative mobile genetic elements (49).

Another group of plasmids that have been extensively studied are the pCAR plasmids that encode genes for the degradation of carbazole/dioxin. pCAR plasmids that have been fully sequenced include pCAR1 from *Pseudomonas resinovorans* CA10 (50), pCAR2 from *P. putida* HS01 (51), and pCAR3 from *Sphingomonas* sp. KA1 (52). Enzymes encoded by the *car* operon transform carbazole to anthranilate, and the proteins encoded by the *ant* operon are responsible for the conversion of anthranilate to catechol (Fig. 2). Carbazole degradation starts with the angular deoxygenation of the compound by the CARDO complex, a three-component dioxygenase that has been extensively characterized (53, 54, 55). In pCAR1, the *car* and *ant* operons are found within a 72.8-kbp transposon named Tn4676 whose transposon-related genes show homology with Tn4651 of the TOL plasmid pWW0. After the introduction of plasmid pCAR1 plasmid into *P. fluorescens* Pf0-1 many rearrangements were observed; one of these is the insertion of Tn4676 into the chromosome (56). Numerous mobile genetic elements are found within the Tn4676 transposon and its flanking region (50). Insertion sequences identical to IS1162 (57), with homology to ISEc8 (58), IS1491 (59), and ISPRe (60) are also encoded in pCAR1. The pCAR3 plasmid is bigger than pCAR1 (254,797 versus 199,035 bp); encoded in pCAR3 are the *car* and *and* operons, as well as a second cluster of *car* genes (*car*-II) and putative genes for the degradation of catechol, protocatechuate, and phthalate. Five different types of insertion sequences and transposons were identified in pCAR3. The backbone of this plasmid is similar to that of pNL1 of *Novosphingobium aromaticivorans* F199 and, although it encoded all the functions necessary for conjugation, attempts to transfer it to a pCAR3-cured strain have been unsuccessful (52). Plasmid pARUE113 (pAL1) from the *Actinobacteria* *Arthrobacter* sp. Rue61a encodes genes for the degradation of quinaldine (61). Quinaldine is transformed to anthranilate through the enzymes encoded by the *meqABC* (*qoxLMS*) operon that encodes quinaldine 4-oxidase and the divergently transcribed *meqDEF* (formerly named *moq, bod*, and *amp*) (62, 63) (Fig. 2). The plasmid also contains a lower pathway for the degradation of anthranilate through CoA intermediates to produce 2-amino-5-oxo-cyclohex-1-ene-carbonyl-CoA. This product is then thought to be degraded via a β-oxidation-like pathway encoded in the chromosome (63, 64). The enzymes of the pathway are induced in the presence of the substrate, and two putative transcriptional regulators, belonging to the GntR superfamily, are located near the catabolic cluster involved in quinaldine degradation. The function of MeqR1 is currently unknown, while MeqR2 is able to bind the promoter region of *meqC, meqD, orf1*, and to its own promoter and shows high binding specificity for anthraniloyl-CoA. These results suggest that MeqR2 may be involved in the regulation of the pathway, although other regulatory systems exist that are known to govern the expression of the *meq* genes (65). Although conserved gene clustering has been observed in pAL1, suggesting a modular structure, only one insertion sequence has been detected (63). Interestingly, pAL1 is a conjugative linear plasmid (61). It has been suggested that the replication of linear plasmids proceeds bidirectionally from an internal origin toward the telomerases generating replicative intermediates that contain 3′-strand overhangs (66). The left and right ends of pAL1 contain palindromic sequences that could be important for telomere patching; furthermore, pAL1 encodes putative proteins that could also be associated with the telomere patching.

**FIGURE 1** Degradation pathways of mono- and biaromatic compounds. Major intermediates of the pathways are depicted. Genes or operons in different plasmids are colored to indicate their role: blue for toluene degradation genes, pink for naphthalene degradation genes, and yellow for biphenyl degradation genes. In green are the genes that can function in different degradation pathways. Genes and operons are not drawn to scale. Operon organization in some cases has not been experimentally demonstrated. doi:10.1128/microbiolspec.PLAS-0013-2013.f1
**FIGURE 2** Degradation pathways for heteroaromatic compounds. Major intermediates of the pathways are depicted. Genes or operons in different plasmids are shown in different colors. Genes and operons are not drawn to scale.
Plasmids That Encode Pathways for the Degradation of Chlorinated Compounds

Catabolic pathways for the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D), a xenobiotic herbicide, have been studied for almost 30 years. One of the best characterized pathways for the degradation of this herbicide is the pathway encoded by the pJP4 plasmid from Wautersia etropha JMP134 (formerly Ralstonia etropha), a strain that is able to mineralize 2,4-D and 3-chlorobenzoic acid (67) (Fig. 2). Two chlorocatechol-degrading gene clusters (tfdI and tfdII) are required for efficient degradation of the compounds (68, 69). The IncP1-β conjugative plasmid pJP4 was sequenced in 2004 (70) and was reported to contain inactive transposons and remnants of lateral gene transfer events. A putative ISJP4-based transposon encompassing the tfdII gene cluster and a putative IS1071-based transposon that flanks all the catabolic genes are found in the pJP4 sequence. However, it has been suggested that these putative transposons are not active (70). The pEST4011 plasmid from Achromobacter xylosoxidans subsp. denitrificans also carries genes for the degradation of 2,4-D (71). Based on differences among backbone proteins of the IncP1 group, it has been hypothesized that this plasmid belongs to a new category, known as the δ subgroup. Within this group, degradative genes are inserted into the backbone at a 48-kbp catabolic transposon that is very similar to Tn5530, which was identified in plasmid pJB1 from Burkholderia cepacia 2a (72). The tfd operon is quite similar to tfdII of pJP4, suggesting a common origin, although tfdD and tfdF have been deleted in the pEST4011 operon (Fig. 2). These genes could have been recruited during evolution to replace the genes lost from the original operon.

Chlorobenzoates are intermediates within the bacterial degradation of polychlorinated biphenyls. Plasmid pA81, from A. xylosoxidans A8 is an IncP1-β plasmid that harbors genes for the degradation of ortho-substituted chlorobenzoates (obhRAB, mochRABCD) in a class I transposon named TnAxl (73). This transposon also carries an operon predicted to function in salicylate degradation, known as bybRABCD (74) (Fig. 3). The pA81 plasmid carries another transposon, known as TnAxlII, that is involved in heavy metal resistance.

Atrazine is another widespread herbicide that many bacteria are able to degrade (75). Genes for the degradation of atrazine in Pseudomonas sp. ADP are located in the 109-kbp pADP-1 plasmid, which was sequenced in 2001 (76, 77). atzA, atzB, and atzC encode the enzymes for the transformation of atrazine to cyanuric acid. Each of these genes is flanked by elements with homology to IS1071, suggesting that they may have been acquired at different times by independent transposition events. The atzDEF operon encodes enzymes that convert cyanuric acid to ammonia and carbon dioxide (Fig. 3). The plasmid belongs to the IncPβ plasmid and has full capabilities for replication, stable maintenance, and conjugation (76, 77).

Chloroaniline degradation is linked to the presence of plasmids pWDL7::rfp (a derivative of plasmid pWDL7 of Comamonas testosteroni strain WDL7) and pNB8c (from Delftia acidovorans strain B8c). Contained within these plasmids are genes for the upper pathway (dcaQTA1A2BR) of chloroaniline degradation, which mediates its transformation to chlorocatechol. This product is then converted to tricarboxylic acid (TCA) intermediates by modified ortho- or meta-pathways encoded by the chromosomes. Both plasmids belong to the IncP-1β incompatibility group and their sequences are very similar, although pWDL7::rfp contains two Tn6063 transposons that carry dcaQTA1A2BR genes (78). These plasmids can be transferred between Beta-proteobacteria and Gammaproteobacteria indicating a broad host range. Transfer of plasmid pNB8c into Cupriavidus pinatubonensis JMP228 confers upon the strain the ability to degrade anilines but not chloroanilines. This deficiency is due to the lack of induction of the 3-CA pathway in this strain. Sequencing of the two plasmids allowed the identification of a difference in the dca promoter region responsible for this lack of induction (78).

Chlorinated nitroaromatic compounds are among the most difficult compounds to degrade because of the electron-withdrawing properties of the nitro and chloro groups. However, some genes associated with chloronitrobenzene degradation are encoded in the pCNB-1 plasmid of Comamonas sp. CNB-1 (79, 80, 81). The degradation pathway converts chloronitrobenzene into 2-amino-5-chloromuconate, which is then degraded to TCA intermediates (Fig. 3). The cnb genes, together with an operon that encodes for resistance to chromate and arsenate are contained within a large transposon belonging to the class I transposon named TnCNB1 (82). pCNB-1 contains 45 open reading frames (ORFs) that provide capabilities for replication, transfer and stability, and similar to other catabolic plasmids, belongs to the IncP1-β incompatibility group.

Other Catabolic Genes Located in Plasmids

A number of genes associated with degradation of lindane (γ-hexachlorocyclohexane) have been found on plasmids. The lin genes of Sphingobium japonicum

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UT26, which transform lindane to β-ketoadiapte, are located in three replicons, with \textit{linDE} (for the conversion of 2,5-dichlorohydroquinone to maleylacetate) and the regulatory gene \textit{linR} found within the 185-kbp conjugative pCHQ1 plasmid \cite{83}. \textit{linB} (which encodes a halidohydrolase) has been found within the 66-kbp pLB1 plasmid \cite{84}. Interestingly, two copies of the gene are located within an IS6100 composite transposon \cite{84}. Based on its homology with the RepA proteins of other plasmids, pLB1 may represent a new plasmid incompatibility group. It is noteworthy that pLB1 was isolated from an unknown bacteria via an “exogenous plasmid isolation” technique, which is used to capture plasmids directly from the environmental microbial community \cite{85}.

The \textit{tsaMVC\textsubscript{D}1}, \textit{tsaQ\textsubscript{1}}, and \textit{tsaT} operons, together with the \textit{tsaMBCD} operon encoding proteins involved in the degradation of p-toluensulfonate in \textit{C. testosteroni} T-2, are linked to the IncP1-β conjugative plasmid known as pTSA \cite{86,87}. The \textit{tsaMBCD} operon contains...
no promoter-like sequence and is not expressed. The *tsa* region forms part of a composite transposon that is flanked by two IS1071 elements. This transposon has been found in several bacterial strains that were isolated from samples enriched in toluenesulfonate on three different continents and was found to be inserted within plasmids or chromosomes (87).

Degradation of phenol has been linked with the presence of plasmids in *Pseudomonas* sp. EST1001 and *Pseudomonas* sp. CF600. The Phe* plasmid of strain EST1001 allows it to grow on phenol (88, 89) and, although it has not been completely sequenced, it is known that the *pheAB* operon is flanked by two IS elements that are involved in the activation of phenol genes (90) and in horizontal gene transfer (91). *Pseudomonas* sp. CF600 harbors the pV1150 megaplasmid that belongs to the IncP-2 incompatibility group and encodes all the genetic information for the degradation of phenol, cresols, and 3,4-dimethylphenol (92, 93).

The alkane degradation pathway encoded in the OCT plasmid of *P. putida* GPo1 is organized into two operons, *alkBFGHJKL* and *alkST*. The first operon encodes all, except for one, of the enzymes required for the terminal oxidation of alkanes to their corresponding fatty acids. The *alkST* cluster codes for the transcriptional regulator AlkS and for a rubredoxin reductase (AlkT) that is part of the alkane hydroxylase complex (94, 95). The OCT plasmid was first described in the 1970s, and its operon expression has served as a model for the study of global regulation responses (96). The two *alk* operons are flanked by ISP*pu4*, forming a class I transposon.

**TOLERANCE PLASMIDS IN BIODEGRADATION**

Although catabolic genes can contribute to the survival of the bacteria in the presence of contaminants, when these contaminants are present at high concentrations, additional mechanisms—in addition to the degradation of the compounds—are required to avoid toxicity. This is the case with organic solvents, which are highly hydrophobic and accumulate in and disrupt cell membranes causing cellular death (97). If the toxic compounds are not degradable (i.e., heavy metals), survival is provided by the action of efflux pumps that eliminate the compounds from the cytoplasm or the membranes of the microorganisms. These mechanisms are normally known as tolerance or resistance mechanisms and many of them are also encoded in plasmids, although, as is sometimes the case with catabolic genes, they can also be located on the chromosome.

**Plasmids Involved in Solvent/Aromatic Compound Tolerance**

*P. putida* DOT-T1E, a strain isolated from a seawater treatment plant in Granada, provides a well-studied example of survival in extreme conditions. This strain is able to degrade different aromatic compounds including toluene (98), and to survive at solvent concentrations up to 90% (vol/vol) (99). The genes that encode the toluene degradation pathway are located on the chromosome (100, 101), while its ability to survive in the presence of high concentrations of toluene is enabled by a 133-kbp plasmid named pGRT1 (102). Solvent tolerance is a multifactorial process that involves a wide range of physiological changes to overcome solvent damage (103); however, mutational experiments have demonstrated that the TtgGHI efflux pump is the most important determinant of solvent tolerance (102, 104). The *ttgGHI* operon of the pGRT1 plasmid encodes an efflux pump of the RND family (resistance, nodulation, cell division) that extrudes a wide variety of compounds, including antibiotics, although not all of them with the same efficiency (104). This family of efflux pumps has been extensively studied because they confer multidrug resistance to some relevant clinical strains (105, 106). The efflux system is formed by three components: an efflux pump transporter, located in the cytoplasmic membrane that recognizes substrates in the periplasm or in the cytoplasmic membrane (107, 108); an outer membrane protein that forms a trimeric channel capable of penetrating into the periplasm and contacting directly with the efflux pump transporter (109); and a lipoprotein anchored to the inner membrane that expands into the periplasmic space and may serve as a bracket for the other two components (110, 111). In the pGRT1 system, the *ttgH* gene encodes for the efflux pump, *ttgI* for the outer membrane protein, and *ttgG* for the periplasmic adaptor protein. Two other RND efflux pumps, chromosomally encoded, have been shown to participate in solvent tolerance in *P. putida* strain DOT-T1E, TtgABC (112), and TtgDEF (113). Despite the contribution of TtgABC and TtgDEF to the solvent-tolerant phenotype, the loss of the TtgGHI efflux pump renders the cells unable to survive in the presence of high concentrations of toluene (102, 104).

The pGRT1 plasmid sequence confirms that it is not a catabolic plasmid (27); however, it shares many features typical of catabolic plasmids. First, the toluene tolerance genes are located within a Tn4653-like transposon (Fig. 4A), which is similar to those found in TOL and pCAR plasmids. This transposon also contains *ttgV*, which encodes the regulatory protein that controls the expression of the efflux pumps (114), two genes that
encode methyl-accepting chemotaxis proteins (mcpT1 and mcpT2), and a gene that is homologous to uvrD. The two MCP proteins are almost identical and allow this bacterium to move toward a broad variety of aromatic compounds and crude oil (115). Monocopies of McpT homologues (99% sequence identity) have been found on other catabolic plasmids of hydrocarbon-degrading strains like pCAR1 of P. resinovorans (116), the TOL plasmid pWW53 of P. putida (22), and the pMAQU02 plasmid of Marinobacter aquaeolei VT8 (NC_008739). All of these strains showed a chemotactic response to toluene. Other examples of MCPs located on plasmids are the NahY protein located on the NAH7 catabolic plasmid of P. putida that responds to naphthalene (35) and NbY from P. fluorescens KU-7 that responds to 2-nitrobenzoate (117). UvrD is a DNA helicase that regulates the activity of the ruvA and ruvB genes, which are present on the pGRT1 plasmid (Fig. 4), and which is involved in the repair of DNA cross-links produced by exposure to UV light and by exposure to toluene (118). UV-resistance genes are located within a broad set of catabolic plasmids. Examples of catabolic plasmids bearing close homologues to the uvrD, ruvA, and ruvB genes from pGRT1 are the naphthalene-degradative plasmids pND071, pDTG1, and pND6-1 (37, 38). One homologue to the universal stress protein (UspA) of E. coli (122, 123) is also encoded in this pGRT1 fragment. It was demonstrated that a pGRT1 mutant in UspA was more sensitive to UV light than wild type, indicating that UspA has a role in the cells’ response to UV stress, possibly in cooperation with UvrD, RuvA, and RuvB homologues (27). The presence of the pGRT1 plasmid was also required for the release of siderophores into the media in response to iron deficiency. This phenotype was linked to the presence of ORF35, which is also encoded by pGRT1. Surprisingly, a BLAST search using ORF35 returns a putative and chromosomally encoded sulfate permease (sulP) found within Pseudomonas and Burkholderia strains (27), although one homologue of this protein has been identified in the antibiotic resistance plasmid pOZ176 of P. aeruginosa 96 (124).

FIGURE 4 Schematic representation of Tn4653-like region (A) and second region encoding stress resistance genes in pGRT1 (B). In red are indicated transposition-related functions, and in blue are putative recombinases or integrases. doi:10.1128/microbiolspec.PLAS-0013-2013.f4
pGRT1 was the first plasmid that was identified to confer cells with solvent tolerant traits. Genes with high sequence homology to those of the ttgGHI operon have been found in other Pseudomonas strains but they are located either within the chromosome (L. Molina, A. Segura, Z. Udaondo, C. Molina-Santiago, J.L. Ramos, unpublished results) or are at undefined locations (125). Because the spectrum of substrates transported by the efflux pump is so wide and extrusion is so effective, plasmid pGRT1 has been considered a paradigm and a model for solvent tolerance. Furthermore, pGRT1 encodes determinants for solvent tolerance, strong chemotaxis toward pollutants, survival under different stress conditions, and genetic transfer to other Pseudomonas strains, thus providing degrader bacteria the ability to survive in heterogeneously polluted environments and to achieve more efficient in situ bioremediation (126). The localization of the efflux pump genes in a transposon and the multiple residues of transposases, recombinases, and integrases found in pGRT1 suggest that genes associated with stress endurance have been recruited from different origins during evolution.

Although TtgGHI is an extreme example of the contributions of an efflux pump toward the resistance to high concentrations of aromatic compounds, many other bacteria have developed systems to cope with toxic compounds present in their niche. An example is the nitrogen-fixing rhizobia bacteria, which are well adapted to live in polyphenol-rich environments. Polyphenols are produced by plants under both biotic and abiotic stresses such as water stress, bacterial or fungal infections, UV radiation, and others (127). Some of the polyphenols exuded by legume seeds and roots are necessary to establish a symbiotic association between rhizobia with their host plant (reviewed in reference 128). However, rhizobia have also developed several mechanisms to thrive in the presence of otherwise toxic concentrations of these compounds, including degradation (129) and tolerance (130). One of the tolerance mechanisms in Rhizobium etli CFN42 is mediated by the RmrAB efflux pump that is encoded by one of the strain’s seven plasmids (131). The rhizobium multi-resistance genes (rmrAB) of R. etli are located within the 184-kbp plasmid known as p42b. This operon is induced by bean exudates and is able to extrude naringenin (a flavonoid inducer of nodulation), coumaric, and salicylic acid, a well-known inducer of plant defense mechanisms that is important for the survival of R. etli in rhizospheric environments (130).

While extrusion of the toxic compounds forms part of the defensive mechanisms against pollutants, stress response mechanisms also play a role. Heat shock proteins (HSPs), ubiquitous proteins that are the key players in the general stress response system, act by mediating the folding and transport of proteins. Organic solvents cause protein damage leading to misfolding. It has been demonstrated that overexpression of heat shock proteins, such as the GroESL system in Clostridium acetobutylicum (a butanol-producing bacteria), results in improved solvent tolerance and solvent production (132). HSP genes can be found in a broad range of antibiotic-resistant plasmids from the Enterobacteriaceae family and in plasmids involved in rhizobia-plant symbiosis. The ORFs known as groES and groEL encode small heat shock proteins that in Rhizobium sp. NGR234 are located within the pNGR234b plasmid (133). Interestingly, genes involved in the degradation of protocatechuic, opine, and naphthalene are also encoded in this plasmid as well as 26 integrases and recombinases. Fragments with different guanine and cytosine (G+C) content have been identified, suggesting that lateral transfer of genetic material may have occurred. The byi_1p (BY123_D) plasmid of Burkholderia sp. Y123 also contains genes for heat shock proteins. This strain was isolated because it was able to degrade the organophosphorus insecticide fenitrothion. With three chromosomes and three plasmids, the strain has degradative genes located in plasmid byi_2p (BY123_E) and byi_3p (BY123_F) (134). Whether or not the heat shock proteins identified within this strain are involved in tolerance has not yet been investigated.

**Plasmids Involved in Heavy Metal Tolerance**

Metal-containing minerals are abundant on Earth. Natural events such as volcanic emissions, forest fires, deep-sea vents, and geysers, together with anthropogenic activities (mining, smelting, the creation of industrial chemical waste, etc.) have contributed to the distribution of these metals across the world. Some metals are necessary to sustain life (calcium and sodium are essential micronutrients; cobalt, copper, nickel, and zinc are vital cofactors for enzymes and metalloproteins); however, at high concentrations they can have toxic effects on the organisms. Other nonessential heavy metals, such as mercury, lead, and cadmium are considered toxic at any concentration (135). Most organisms have developed homeostasis systems in order to maintain optimal intracellular concentrations of metals. This is achieved through the control of the processes of transport (import and export) and intracellular trafficking to prevent cellular damage and ensure cellular bioavailability. Metal transporters move metal ions or chelates through membranes, metallochaperones transfer the metal to appropriate
cellular compartments or acceptor proteins, and efflux pumps can eliminate excess and unwanted metals. Regulatory proteins serve to control the expression of all these proteins in response to metal deprivation or overload (136). While normal heavy metal concentrations in soils are generally nontoxic, there are specific locations where metal concentrations are high enough to prevent “normal” organisms from existing (137) and where only organisms with special mechanisms of tolerance can thrive. Efflux pumps are one of the main mechanisms for metal resistance in several bacteria, but metal complexation and metal reduction can also contribute to tolerance (133).

Cupriavidus metallidurans CH34 has served as a model microorganism for heavy metal resistance. The bacterium was isolated from the sludge from a zinc decantation tank in Belgium (138) and tolerates high concentrations of heavy metal ions, including but not necessarily limited to Cu⁺, Cu²⁺, Ni²⁺, Zn²⁺, Co²⁺, Cd²⁺, CrO₄²⁻, Pb²⁺, Ag⁺, Au⁺, Au³⁺, HAsO₄²⁻, AsO₄³⁻, Hg²⁺, Cs⁺, Br⁻, Cl⁻, Ti⁴⁺, SeO₄³⁻, SeO₃²⁻, and Sr²⁺. This strain is a facultative chemolithoautotroph that is able to grow using toluene, benzene, or xylene as the sole carbon source. This ability is conferred by a number of chromosomally encoded genes as well as uncharacterized dioxygenases located on plasmids. The genome of C. metallidurans CH34 is composed of four replications: two main chromosomes and two megaplasmids named pMOL28 and pMOL30 (135, 139). This strain accomplishes metal detoxification via the action of a wide variety of efflux systems of the RND, P-type ATPase, and Cation Diffusion Facilitation (CDF) families. It also contains transporters that are specific to certain metal ions, whereby efflux may be followed by metal sequestration or complexation. Many of these systems are localized in two large plasmids known as pMOL28 and pMOL30 (135).

The pMOL28 plasmid (139) has been associated with tolerance to Ni(II), Co(II), CrO₄²⁻, and Hg(II). The nickel/cobalt tolerance determinant present in pMOL28 is the enr operon, which is composed of the RND efflux pump CnrABC, and a regulatory complex formed by the regulatory proteins CnrXY and the sigma factor, CnrH. Additionally, CnrT, a cation diffusion facilitator, is encoded by this region. Cation diffusion facilitators are transport systems driven by a chemiosmotic gradient (140) and represent another line of defense against excess metal cations. These proteins form a family of membrane-bound secondary transport systems for divalent transition metal cations (141). The Cupriavidus CH34 strain contains two additional CDF proteins, known as DmeF and FieF (140), that are chromosomally encoded (142). Chromate tolerance is mediated by the efflux pump ChrA, which belongs to the chromate-efflux-related protein family. These systems are driven by proton-motive force and remove chromate by efflux in cooperation with other proteins (143). Proteins related to this efflux pump are the regulatory proteins ChrF, ChrI, and ChrB; ChrE, which is involved in the processing of chromium-glutathione complexes; and ChrC, an iron superoxide dismutase (Fe-SOD). Homologues of the chr operon have also been found in a plasmid of the potential PAH degrader Arthrobacter sp. FB24 (144); in pCNB1, which provides Comamonas CNB-1 the ability to degrade chloronitrobenzenes (82); and in the pRA4000 plasmid, which allows P. putida NCIMB 9866 to degrade 2,4-xylenol and p-cresol (145).

Mercury resistance is widespread in plasmids. Although mercury concentration in soils and waters is normally very low, since the start of the industrial revolution, the amount of mercury mobilized and released into the biosphere has increased, and, in some places, local mercury levels have increased by several thousandfold above ground. The heightened levels of mercury in the environment may be responsible for the widespread occurrence of the mer operon in nature (146). In pMOL28, mercury resistance is associated with the presence of the merRTPADE operon (147). MerR acts as the key regulator of this operon (148). The highly toxic Hg²⁺ cation is bound in the periplasm by MerP, imported into the cytoplasm by MerT, and reduced to metallic mercury by the MerA NAPDH-dependent flavoprotein. While these enzymes are essential for mercury detoxification, in some organisms the operon contains additional genes including merB, which encodes an organomercurial lyase; merC, a mercury transporter; and MerD, which is involved in regulation of the mer operon (reviewed in reference 149). The presence of the mer operon in other plasmids has been associated with other degradative pathways. Examples include the pW2 plasmid from P. putida W2 (NC_013176), involved in bisphenol A degradation; the byi_1p plasmid from Burkholderia sp. Y123, that degrades fenitrothion (134); the pL2 plasmid from Comamonadaceae for the degradation of aniline (150); and in the pJP4 plasmid from R. eutropha JMP134, which allows degradation of chloroaromatics (70).

The backbone genes of the pMOL28 plasmid are highly homologous with the pHG1 plasmid core genes, which contain essential genes required for the facultative lithoautotrophic and facultative anaerobic lifestyles of R. eutropha H16 (151) and pSym of Rhizobium taiwanensis LMG19424 (152). The presence of one
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copVTMKNS1R1A1B1C1D1I}GFLQHE in which CopF is the P-type ATPase involved in Cu(II) efflux from the cytoplasm. CopT is believed to be involved in metal transport from the periplasm to the cytoplasm; CopR and CopS are members of the two-component family of sensor regulators; CopA is a multicopper oxidase; CopB is thought to be involved in Cu(I) fixation; CopC contains binding sites for Cu(I) and Cu(II) and may be involved in detoxification of both ions; copD encodes a putative channel involved in loading CopA with Cu(II); CopI is a putative oxidoreductase; CopJ showed similarity with cytochrome c proteins; CopL may have a role in regulation; and CopH has homology with czeE. No function has been assigned to CopV, CopK, CopM, CopN, CopG, CopQ, and CopE (157). The cop system is responsible for copper and silver resistance of E. coli (158). Homologous cop genes have been identified in the metal tolerance plasmid pSPHC01 of Sphingobium chlorophenolicum L-1 (159), and in the plasmid pILSP0 from Sphingomonas sp. MM-1, which also harbors genes involved in the degradation of γ-hexachlorocyclohexane (160). Homologues of copA, mco (multicopper oxidase gene), cadD, and cadX (cadmium resistance) have also been recently identified in multiresistance plasmids from Staphylococcus aureus ST398 (161). The colocalization of antimicrobial resistance genes and genes that confer tolerance to heavy metals can facilitate the persistence and dissemination of these traits and could pose a serious problem for human health. Because of the widespread use of copper bactericides to control bacterial infections in crop plants, the copABCD system (present in the chromosome of C. metallidurans CH34 and sharing low sequence homology with the cop operon of plasmid pMOL30) and the cusCBA operon are found in plasmids of P. syringae pv. tomato (162) and pv. syringae UMAP0081 (163). The second P-type ATPase system present in pMOL30 is the lead resistance operon, pbr, which contains the following structural genes: pbrT, which encodes a Pb(II) uptake protein; pbrA, which encodes a P-type Pb(II) efflux ATPase; pbrB, which encodes a predicted integral membrane protein of unknown function; and pbrC, which encodes a predicted prolipoprotein signal peptidase. Downstream of pbrC, the pbrD gene encodes a Pb(II)-binding protein. The PpbrA promoter is regulated by PbrR, which belongs to the MerR family of metal ion-sensing regulatory proteins. This operon is also found in antibiotic/heavy metal tolerance plasmids of Enterobacter cloacae and Klebsiella pneumoniae strain KCTC 2242 (164).

pMOL30 parAB genes are very similar to those in B. vietnamiensis G4, and belong to a different incompatibility group than pMOL28, as they are stably

genomic island in pMOL28 that contains all the determinants for heavy metal resistance has been described (139). This region is flanked by IS1071 and a partial IS from the Tn3 family.

The pMOL30 plasmid (234-kbp) encodes determinants for tolerance to Ag(I), Cd(II), Co(II), Cu(II), Hg(II), Pb(II), and Zn(II) (139). In addition to containing a mer operon that is similar to that found in pMOL28, pMOL30 contains the czeABC operon (one of the best studied zinc-tolerance systems), which encodes an RND efflux pump for cobalt, zinc, and cadmium resistance in C. metallidurans (140). The czeABC operon includes three components of the proton antiporter efflux system: czeD, which encodes a cation diffusion facilitator; and czeSR, which encodes a two-component regulatory system. These regulators are homologues to those involved in copper resistance, and it is likely that this operon mediates copper extrusion (139). Three additional proteins, CzeN, CzeI, and CzeCE, are putative components of the resistance mechanisms, although their function is currently unknown. Extrusion systems that are homologous to cze have been found in catabolic plasmids such as pCAR3 of Sphingomonas CH34 and sharing low sequence homology with the cze operon of plasmid pTOM9 from C. metallidurans (of pMOL28) and cusCBA operon are also encoded by pMOL30. The cnu (of pMOL28) and ncc systems are related to the NccA protein of the nickel-cobalt-cadmium determinant of the pTOM9 plasmid from Achromobacter sp. 31A (155). The silcux system encoded by pMOL30 may be involved in the efflux of silver and copper ions. The silABC operon encodes an efflux pump for the transport of these ions (140). Other factors that are not found on the pMOL30 plasmid include: SilE, a protein that acts as an extracellular metal-binding protein; and silRS, which encodes a two-component sensor and transcriptional responder. The silRS operon is widespread in plasmids originating from clinical strains probably because silver compounds are used as antimicrobial agents against bacterial infections (156).

Two of the 13 predicted P-type ATPases of C. metallidurans CH34 are present in pMOL30. P-type ATPases constitute a ubiquitous superfamily of transport proteins that are driven by ATP hydrolysis. Among their substrates are inorganic cations such as H^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Cu^+, Ag^+, Zn^{2+}, and Cd^{2+}. In contrast to RND pumps, these ATPases are also able to detoxify heavy metal cations bound to thiols. One of the P-type ATPases of pMOL30 is encoded by the large cluster
maintained. pMOL30 was found to be able to transfer at very low frequency. Many recombinases, IS, and truncated IS have been identified in pMOL30, most of them close to tolerance determinants. The zce and pbr clusters are flanked by the mercury transposon Tn4380 on one side and by three mer genes that might be remnants of former rearrangements. The region containing the cop, sil, and mre-ncc clusters is flanked by a complete ISRme10 element and a remnant of another named ISRme10 (139).

Resistance to the toxic divalent heavy metal cations of cobalt, nickel, cadmium, and zinc is widespread among bacteria, although in many of them the genetic determinants of this characteristic are associated with chromosomal genes (165). As mentioned above, C. metallidurans CH34 also has many tolerance determinants located on the chromosome.

Resistance to arsenite [As(III), As(OH)3], arsenate (As(V), AsO4−3), and antimony [Sb(III)] is widely found among Gram-negative and Gram-positive bacteria. Usually this resistance is determined by the presence of an ars operon with a minimum of three cotranscribed genes that include arsR (a regulatory repressor), arsB (a membrane transport pump), and arsC (a small intracellular arsenate reductase). Additionally, in this operon other proteins are encoded, including: ArsA, which is an ATPase coupled to ArsB (together, these increase arsenate resistance); ArsD, which acts as an arsenite chaperon; and ArsP, which is a putative membrane permease (166). Although the ars operon is mostly located chromosomally, components are also distributed in plasmids, especially those encoding antibiotic resistance (167). One example of ars operon within a plasmid is found in Arthrobacter sp. Rue61, which degrades quinaldine through a pathway encoded by the pARUE113 (pAL1) linear plasmid. This strain also has a circular plasmid, named pARUE232, which harbors ars genes (64). Another example of a plasmid that has the ars operon is the metal tolerance plasmid pOC167 from Oligotropha carboxidovorans OM3 (168).

**GENOMIC BIOAUGMENTATION AS A BIOTECHNOLOGICAL APPLICATION**

The existence of the described solvent, heavy metal tolerance, and catabolic plasmids provides a useful battery of biotechnological tools for remediation of polluted environments. Contaminants are normally present in complex mixtures that make degradation difficult. Some of these compounds, as described earlier, are toxic and are able to avoid the action of the catabolic pathways of pollutant degrader microorganisms. Tolerance plasmids and genes, once introduced into degrader strains, will allow the survival and the metabolic activity of bacteria living in polluted environments. One of the strategies to bioremediate contaminated environments is bioaugmentation with strains that have the capacity to degrade pollutants. However, it has been observed that laboratory strains, on many occasions, were not able to thrive in the new environment, leading to unsuccessful bioremediation (169). Given the possibilities of plasmid exchange among strains, bioaugmentation in catabolic genes, not in bacteria, has taken relevance in the past decade (170). This approach is called “genetic bioaugmentation” and involves the introduction of bacteria harboring a relevant catabolic self-transmissible plasmid that stimulates the horizontal gene transfer of the plasmids into indigenous microorganisms with better fitness for survival in the corresponding niche (171). To design an optimal genetic bioaugmentation is important to choose the appropriate type of plasmid, to study the transfer capacity of the microorganism and the stability of the plasmid in the new bacteria, as well as to study the expression of the catabolic genes (171, 172). Plasmid stability in the recipient strains may impact the effectiveness of bioremediation; highly stable plasmids could be necessary to clean up sites that continuously receive contaminant input (171). Horizontal gene transfer from donor to indigenous bacteria following the deliberate release of phenol-degrading laboratory bacteria was shown to be important in the degradation of phenol in river waters continuously polluted by phenolic compounds (91).

It is well documented that the TOL plasmid can be transferred from P. putida to other *Pseudomonas* and *Erwinia* strains (173), and to *Enterobacteriaceae* (174). Ikuma et al. (175) showed that the soil organic carbon present in sterilized soil slurries was sufficient for transfer of TOL plasmid using different mixtures of recipient bacteria (including *E. coli*, *E. cloacae*, *Serratia marcescens*, *P. fluorescens*, and *P. putida* BBC443). However, they also observed that addition of glucose sometimes improved the specific toluene degradation rates of *Enterobacteriaceae* transconjugants. The G+C genomic content of the recipient strains have a clear influence on the expression of the toluene degradation genes in TOL transconjugants and the presence of alternative carbon sources (such as glucose) was shown to alleviate the limitations of the expression of the acquired genes in some transconjugants (176). Observations made under laboratory conditions (177) indicated
the importance of the initial recipient-to-donor cell density; however, in the experiments conducted by Ikuma et al. (175) this factor had only a minor impact on plasmid conjugation. The spatial separation between donor and recipient strains can pose a problem for the plasmid transfer and efficiency of biodegradation; this problem has been tackled with the use of different approaches. The use of earthworms has been reported to increase the dispersal of donor (A. eutrophus [pJP4]) and recipient bacteria (P. fluorescens) and to increase the frequency of transconjugants in soil microcosms (178). Similarly, biofilm structures, with open channels and pores, allow the efficient transport of donor cells facilitating collision between bacteria and enhancing the introduction of mobile elements into an existing microbial community (179). Interestingly, transfer of the TOL plasmid in sequencing batch biofilm reactors (SBBR) used for treating synthetic wastewater containing benzyl alcohol was observed on a laboratory scale but not in the pilot scale bioreactor (180). This failure can be attributed to different operational conditions in the two bioreactors and to the lack of selective pressure in the pilot scale bioreactor, because complete benzyl alcohol removal was achieved during the first 60 minutes of operation. While the presence of contaminant has been reported to exert a positive effect on the efficiency of the pJP4 plasmid transfer under nonsterile conditions (181), the presence of toluene did not influence the TOL transfer frequency when tested in filter matings (177) or in slurry soils at environmentally relevant concentrations (176).

Other approaches to bring together donor and recipient bacteria take advantage of the high number of bacteria living near the plant roots; roots additionally provide a solid surface for conjugation (172). Although the rhizosphere can improve the survival of microorganisms in soil (182) and thus promote the elimination of contaminants (183), it is also a complex environment where plant and microorganisms establish different kinds of relationships. Because the rhizosphere is highly populated, microbes have to compete for niches and nutrients (184); furthermore, plants secrete harmful compounds such as phytoalexins, phenolic derivatives, and others. Transcriptional experiments performed with the rhizospheric strain P. putida KT2440 showed that they sense the rhizosphere as a stressful environment (185). Some genetic traits involved in coping with this high-stress environment have been shown to be encoded on plasmids; the best characterized examples are the symbiotic plasmids from Rhizobium strains. These plasmids encode chemotaxis systems that recognize rhizosphere nutrients, genes involved in catabolism of these nutrients, nutrient uptake, attachment to roots, and detoxification of harmful molecules produced by plants (reviewed in reference 186). To avoid competition with other rhizospheric microorganisms, Barac et al. (187) used a new approach; it involved the introduction of the pTOM plasmid into B. cepacia L.S.2.4, a natural endophyte of the legume, Lupinus luteus (yellow lupine). This approach improved the biodegradation of toluene. Inoculation of poplar trees with another yellow lupine endophyte, B. cepacia VM1468 containing the pTOM plasmid, also resulted in decreased toluene toxicity toward the plant. Although B. cepacia VM1468 was not able to successfully establish in the plant at high levels, it was able to transfer the pTOM plasmid to other indigenous endophytes, demonstrating the horizontal transfer of the plasmid (188).

In recent years some “rhizospheric” plasmids have been identified. One of them is plasmid pQBR103 from P. fluorescens SBW25. It belongs to a large group of plasmids known to persist in the sugar beet phytosphere (rhizosphere and phyllosphere) and to be confined within the Pseudomonas group. The pQBR103 is a 425-kbp plasmid that encodes one mercury resistance operon located in Tn5042 type II transposon, and also encodes the RulAB proteins that confer UV light resistance. Field release trials have shown that pQBR103 confers a significant advantage to the SBW25 strain, 3 to 5 months after planting (189). Preliminary studies demonstrated that the advantages provided by this plasmid are imposed by the modulation of plant responses, although the mechanism of the adaptive advantage remains unclear (190). It has been shown that pQBR103 regulates up to 48 proteins encoded on the chromosome of its host strain.

**PERSPECTIVES**

Even though the decreasing costs of sequencing have allowed more information to be obtained about catabolic or tolerance plasmids in the environment, there is still a considerable lack of knowledge regarding plasmids from noncultivable bacteria. Several approaches have been followed to fill this gap. The most commonly used technique has probably been “exogenous plasmid isolation.” In this technique, a cultivable recipient strain is used to acquire plasmids from different environments through conjugation (191, 192, 193, 194, 195). However, transfer is limited by the incompatibility of the indigenous bacteria for establishing interactions with the recipient bacteria and the capacity of
the plasmid to be transferred and replicate in the new strain. To avoid the dependence on the plasmid-encoded traits two different techniques have been described. One of them is called transposon-aided capture (TRACA) of plasmids. Genomic extracts from environmental samples are treated with plasmid-safe DNase that digest linear but not circular DNA, then circular plasmids are subjected to transposition with insertions that contain an E. coli origin of replication and a selectable marker later transformed into E. coli strains (5). This technique has been successfully used for the identification of plasmids resident in the human gut meta-genome but, to date, we have not found any report describing the utilization of this technique in the isolation of catabolic or tolerance plasmids. We can envisage certain drawbacks for TRACA utilization in the identification of catabolic or tolerance plasmids; because of the large size of the catabolic plasmids, the DNA can be physically broken or damaged during the extraction process and thus later on digested by the DNase; also because of this large size transformation into E. coli strains would be difficult. The approach followed by Kav and colleagues (196) is also based on the treatment of genomic environmental DNA with a plasmid-safe DNase, but the resultant circular plasmid DNA is subjected to amplification with DNA polymerase from phage q29 and sequenced. With the use of this technique, the plasmidome of the bovine rumen has been identified (196). Implementation and improvements in both protocols could circumvent the plasmid size issue of catabolic and tolerance plasmids and will allow the analysis of the plasmidome of environmentally relevant niches.

The impact of catabolic plasmids on host cell physiology is another area of research that has to be further developed (review in reference 197), because this information is important for improving the biotechnological utilization of bacteria carrying catabolic genes. It is known that plasmid carriage generally leads to loss of host fitness (198), but catabolic and tolerance plasmids are quite stable in their corresponding natural host even in the absence of selective pressure. The pCAR1 plasmid is stably maintained not only in its natural host, P. resinovorans, but also in P. putida KT2440. The transcriptome of P. putida KT2440 harboring pCAR1 was compared with that of the plasmid-free strain when growing on succinate (116). Interestingly, plasmid pCAR1 did not significantly interfere with the host transcriptional patterns, and only parl, a homologue of the ParA family of plasmid partitioning proteins from a cryptic genomic island, showed a significant induction in expression. It was latter demonstrated that Parl interferes with the IncP-7 plasmid partitioning system (199), so the reason why pCAR1 is stable in KT2440 remains unclear. Transcriptional analysis of pCAR1-carrying and pCAR1-free strains of P. putida KT2440, P. aeruginosa PAO1, and P. fluorescens Pf0-1 was performed by using high-density tiling arrays in order to identify changes in transcriptional patterns. Although 70 to 100 genes (depending on the strain) were found to be altered in their expression levels in the pCAR1-carrying strains compared with the pCAR1-free strains, only four genes were found to be upregulated in the three strains. Three of the genes form an operon that contained a Fur-associated gene (Fur is a global regulator in response to iron limitation); the fourth gene (phuR) is known to be upregulated under iron-limited conditions in P. aeruginosa. Among the commonly regulated genes in two of the hosts, most of them were related to iron acquisition and transport systems. Notably, pCAR1-carrying P. putida KT2440 and P. aeruginosa PAO1 have higher levels of pyoverdine than the plasmid-free cells, which suggests that iron concentration may be key factor in the maintenance of pCAR1 in host cells (200). Backbone-related functions are also important in plasmid survival in different strains; more than 90% of ORFs on the pCAR1 backbone were transcribed in six different host strains (201). The influence of the NAH7 plasmid on the P. putida KT2440 transcriptome has also been analyzed. Despite the fact that the presence of the plasmid relieved the stress caused by the presence of naphthalene in the host strain, few genes were differentially expressed between the containing and noncontaining strains when naphthalene was not provided (202). The effect that nucleoid-associated proteins (NAPs) have on the expression of plasmids and host chromosomal genes has to be further explored. Disruption of Pmr, a histone-like protein H1(H-NS) encoded on pCAR1, significantly alters the expression levels of several genes in P. putida KT2440 (203). The knockout inactivation of TurA, a chromosomally encoded protein with structural similarity to H-NS proteins, resulted in enhanced transcription initiation from the Pu promoter, suggesting a negative regulatory role of TurA on Pu expression (204).

Many different biotechnological applications that rely on plasmid activities in a community are currently being developed for the elimination of contaminants (205). Although plasmid stability is usually high, segregants can become a majority if plasmid-free cells have faster growing rates (206). To monitor the abundance of plasmids, new rapid, noninvasive, in situ monitoring techniques will have to be improved to detect the maintenance of plasmids within the reactors. Traditional
monitoring techniques, such as replica plating, selective markers, and PCR detection (207) have, in general, low sensitivity and, in some cases, they can disrupt the function of the system. Although techniques based on the introduction of gfp-labeled plasmids have been proposed as a noninvasive, in situ monitoring solution (208), the use of recombinant plasmids may carry some legal restrictions in real-life applications.

CONCLUSIONS

Although many catabolic pathways and resistance operons are located on plasmids, these genes can also on many occasions be found on the chromosome. Nevertheless, catabolic and tolerance plasmids that are generally self-transmissible, are valuable tools for bioremediation applications. Although in the 1980s there was avid interest in the discovery of contaminant degradation pathways and their regulatory circuits, it was at the beginning of the 21st century that the simplification of sequencing techniques allowed a better understanding of the plasmids that encode these pathways. It is now known that most of the catabolic and tolerance genes encoded on plasmids are associated with mobile elements, and that transfer to or from the host bacteria has occurred not only as a consequence of plasmid transfer, but also because of transposition and recombination. The backbones of many of these plasmids are related to the IncP incompatibility group, and, in many cases, plasmids share a similar backbone but are loaded with different catabolic or tolerance genes (i.e., the backbone of pBVIE04 that carries the toluene monoxygenase genes and the backbone of pGRT1 that carries the toluene tolerance operon).

There is a lack of information about plasmids in the environment that are harbored by noncultivable strains; this knowledge gap needs to be filled. Awareness of the abundance and significance of these plasmids in the environment will allow us to better understand the function of ecosystems. Understanding the mechanisms behind plasmid stability will improve the outcomes of the biotechnological application of these plasmids.

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