The applicability of genetically modified microorganisms in bioremediation of contaminated environments

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Please cite as: CHEMIK 2012, **66,** 8, 817-826

Introduction

Many industrial and agricultural activities, especially in the last 50 years, have caused the significant increase in the concentration of toxic pollutants in environments. Among man-made substances the most dangerous are chlorophenols, nitrophenols, BTEX (benzene, ethylbenzene, toluene and xylene), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls and organic solvents. The main sources of these compounds in soil, wastewaters and aquifers are coal gasification, coke-oven batteries, refinery, petrochemical plants and other industries, such as synthesis of chemicals, herbicides and pesticides. Most of these substances are mutagenic and carcinogenic, break down slowly and remain in the environment for a long period of time. For this reason, the removal of such toxic chemicals from contaminated areas is constantly of great importance [1, 11, 19, 26].

Nowadays, biological methods in treatment of organic pollutants are recommended. One of the possible, cost-effective and safe technology, which enables to resolve contamination problem is bioremediation. It refers to a process that uses microorganisms and their enzymes to promote degradation and removal of contaminants from the environment. Bioremediation methods are performed either *in situ* (bioaugmentation, biostimulation and bioventing) and *ex situ* (landfarming, biopiles and bioreactors) [9, 26, 27].

Bioremediation techniques use microorganisms because many of them are able to break down contaminants. It is connected with their metabolism that involves biochemical reactions or pathways related to organism activity and growth. Microorganisms can decompose or transform hazardous substances into less toxic metabolites or degrade to non-toxic end products. In the process called "cometabolism" the transformation of contaminants yields little or no benefit to the cell, therefore it is described as a nonbeneficial biotransformation [5, 9, 25].

At the beginning of the 80s the development of genetic engineering techniques and intensive studying of metabolic potential of microorganisms allowed to design genetically modified microorganisms (GMMs). At present, they are applied in a variety of fields such as human health, agriculture, bioremediation and different types of industry [5]. The construction of GMMs, which will be able to degrade organic compounds, is possible because many degradative pathways, enzymes and their respective genes are known and biochemical reactions are well understood. This knowledge gives opportunity to create GMMs with new metabolic pathways. The GMMs may be an alternative solution for wild strains which degrade contaminants slowly or not at all. Innovative approaches are indispensable to decrease the level of toxic organic compounds in environments and maintain their good quality and ecological status [5, 27]. In 1981 in the USA the first two genetically modified strains of *Pseudomonas aeruginosa* (NRRL B-5472) and *Pseudomonas putida* (NRRL B-5473) were patented. They were constructed by Chakrabarty in the early 70's and contained genes for naphthalene, salicylate and camphor degradation [43]. In turn, naphthalene-degrading *Pseudomonas fluorescens* HK44 represents the first genetically engineered microorganism approved for field testing in the USA for bioremediation purposes [33].

This review focuses on the construction and use of GMMs in bioremediation of environments contaminated with organic compounds. It presents several molecular tools and strategies how to create new engineered microorganisms and how use them in environment safely. It is also discussed the risk associated with the release of GMMs into contaminated areas. Additionally, some examples of GMM applications in laboratory and field experiments are presented. In final part of this review, special attention is paid to the legal regulations of genetically modified organisms (GMO).

Construction of GMMs

Genetic engineering is a modern technology, which allows to design microorganisms capable of degrading specific contaminants. It offers opportunity to create artificial combination of genes that do not exist together in nature. The most often techniques used include engineering with single genes or operons, pathway construction and alternations of the sequences of existing genes [5, 8, 18].

The first step in GMM construction is selection of suitable gene/s. Next, the DNA fragment to be cloned is inserted into a vector and introduced into host cells. The modified bacteria are called recombinant cells. The following step is production of multiple gene copies and selection of cells containing recombinant DNA. The final step includes screening for clones with desired DNA inserts and biological properties [7, 8, 18, 28, 39]. The basic stages in molecular cloning illustrates Figure 1.

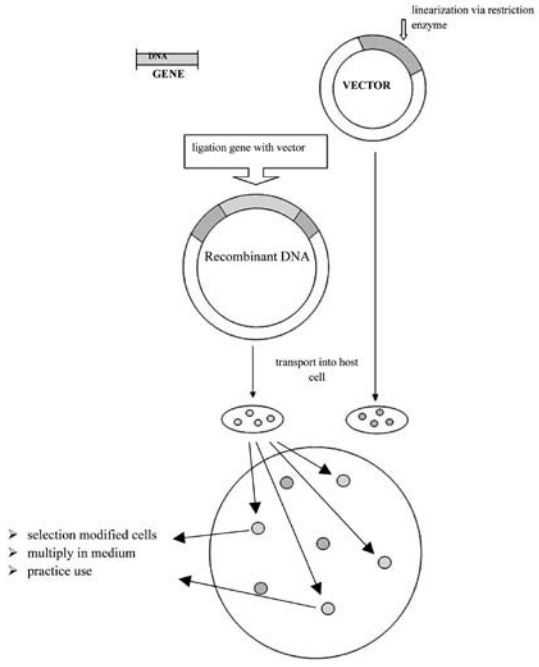

Fig. 1. Steps in molecular cloning (7, 18; modified)

Bacteria, especially from genus *Pseudomonas*, are the major object of genetic manipulations. There are ubiquitous inhabitants of many environment and are known as efficient degraders of many toxic substances. Both their chromosome and plasmids may carry genes for metabolism of these compounds. Therefore, such microorganisms are the main source of catabolic genes for genetic engineering [5, 9]. The first catabolic plasmid TOL (117 bp) from *Pseudomonas putida* mt-2 was described by Williams and Murray [40]. It contains two operons *xylUWCMABN* and *xylXYZLTEGFJQKIHSR* encoding enzymes involved in metabolism of toluene, *m*- and *p*-xylene, and *m*-ethyltoluene. Another catabolic plasmid NAH7 (83 kb) from *P. putida* G7 is a donor of two operons *nah* and *sal*, encoding enzymes for naphthalene and salicylate metabolism [25]. The operon *tod* from *P. putida* F1 is often used in genetic engineering experiments as a source of *todABC1C2DEF* genes responsible for toluene degradation [42]. In turn, biphenyl-utilizing bacteria *Burkholderia cepacia* LB400 can be used as a donor of *bphAEFGBC* genes [23]. Many plasmid-born catabolic genes for the degradation of toxic substances are often located in transposons, for example in Tn*4653* from *P. putida* mt-2, Tn*4655* from *P. putida* G7 and Tn*4656* from *P. putida* MT53 [36].

Plasmids are commonly used as cloning vectors in genetic engineering to multiply or express particular genes. Vector is a genetic molecule for transfer of a new genetic information into another cells, where it replicates independently of their chromosomal DNA. It often contains a set of various genes, for example antibiotic resistance genes. The other genetic elements called transposons could also act as vectors [8, 9, 28]. Nowadays, the artificial plasmid vectors in construction of GMMs are commonly used. They contain the best features derived from different natural plasmids such as oriC (origin of replication), MCS (multi-cloning site) and marker genes. Presently, expression plasmids are widely used because they enable the quick production of a large quantity of desired protein. Apart from the vectors, enzymes as a powerful genetic engineering tool in the cut-and-paste techniques are indispensable. They include restriction enzymes cutting DNA in a specific region and DNA ligases which close nicks in the phosphodiester backbone of DNA. Among them, restriction endonuclease *Eco*RI, *Bam*HI and *Hind*III and T4 DNA ligase are commonly used in molecular biology [7, 8, 18, 38].

For practical reasons, many recombinant vectors were designed. For example, Ouyang et al. [29] constructed plasmid pBBR1MCS-2 harboring 3.9 kb fragment containing *tac* promoter from plasmid pKST11 and *todC1C2BA* genes responsible for toluene degradation. This recombinant DNA was inserted into plasmid's *Bam*HI site to express *tod* genes in *Pseudomonas putida* KT2442, *P. stutzeri* 1317 and *Aeromonas hydrophila* 4AK4. In other study, Chen et al. [6] designed artificial plasmid by cloning *ohb* gene (orthohalobenzoate 1,2-dioxygenase) into vector pSP329 and *lacZ* gene into its *Hae*II site. In turn, Sayler and Ripp [33] used the transposon Tn*4431* as a vector for *lux* genes. Haro and de Lorenzo [14] assembled pathway included one catabolic segment encoding toluene dioxygenase of the TOD system of *P. putida* F1 (*todC1C2BA*) and the second catabolic segment encoded the entire upper TOL pathway from pWW0 plasmid of *P. putida* mt-2. Both TOD and TOL fragments were assembled in separate mini-Tn*5* bacterial transposons and were inserted into chromosome of 2-chlorobenzoate degraders *P. aeruginosa* PA142 and *P. aeruginosa* JB2.

Genetic transfer is the mechanism by which DNA is transferred from a donor to a recipient. In laboratory scale, recombinant bacteria capable of metabolizing toxic organic compounds are usually obtained through transformation. Transformation is gene transfer resulting from the uptake free naked DNA from environment by a competent recipient bacteria cells. The next possibility of DNA transfer by direct physical contact between the cells is conjugation. DNA transfer by conjugation occurs only in one direction, from a donor to a recipient [8, 28]. Ouyang et al. [29] used conjugation as a way of plasmid pKST11 transfer from *Escherichia coli* S17-1 to three recipient strains representing by *P. putida* KT2442, *P. stutzeri* 1317 and *A. hydrophila* 4AK4. Chen et al. [6] transferred plasmid pE43 into *Sinorhizobium meliloti* recipient cells by electrotransformation (low voltage, direct current). In turn, Matsui et al. [24] transformed *Mycobacterium* sp. and competent cells of *E. coli* JM109 with recombinant plasmid pNC950 by electroporation (high voltage pulses of electricity).

Nowadays, for the selection and identification of GMMs modern molecular techniques such as FISH (fluorescent *in situ* hybridization), *in situ* PCR (*in situ* polymerase chain reaction), DGGE (denaturing gradient gel electrophoresis), TGGE (temperature gradient gel electrophoresis), T-RFLP (terminal restriction fragment length polymorphism) and ARDRA (amplified rDNA restriction analysis) are used. These methods are based on detection of specific DNA or RNA sequences, especially conservative fragments in bacterial 16S rRNA [27, 37]. For example, Dejonghe et al. [10] used DGGE for monitoring horizontal transfer of plasmids pEMT1 and pJP4 from engineered donor strain of *P. putida* UWC3 to the indigenous bacteria during degradation of 2,4-dichlorophenoxyacetic acid in soil.

Another way of tracking and visualizing bacteria in environmental samples is marker system. The ideal marker system should enable detection and quantification of specific organisms and allow to monitor the cellular events associated with gene expression and signal transduction. As marker genes *lacZ* (β-galactosidase), *lux* (bacterial luciferin-luciferase system), *tfd* (monooksygenase), *xylE* (catechol 2,3-dioxygenase), *gfp* (green fluorescent protein) are commonly applied [8, 9, 15, 37]. Sayler and Ripp [33] used operon *lux* in plasmid pUTK21 for detection of naphthalene-degrading recombinant strain of *P. fluorescens* HK44 in soil. In other study, Villacieros et al. [38] monitored recombinant *P. fluorescens* F113L::1180 strain by *gfp* gene expression during rhizosphere bioremediation of polychlorinated biphenyls (PCBs). In turn, Quan et al. [31] used marker genes *dsRed* (red fluorescent protein) located in pJP4 plasmid and *gfp* gene inserted into chromosome of *P. putida* SM1443 in detection of recombinant bacteria during 2,4-dichlorophenoxyacetic acid (2,4-D) degradation in bioaugmented soil.

In order to reduce potential risk of the use of GMMs in the environment some genetic barriers are created. They limit the survival of recombinant bacteria and gene transfer. The spread of genes from GMMs to other microorganisms may be limited by using transposons without transposase gene or by removing *tra* conjugation genes from the recombinant plasmid. Random horizontal gene transfer can be also diminished by inserting into vector *colE3* gene encoding colicin that cuts all prokaryotic 16S rRNA and by controlling *immE3* gene encoding repressor of colicin synthesis [9, 20]. The regulation of lethal gene presents Figure 2.

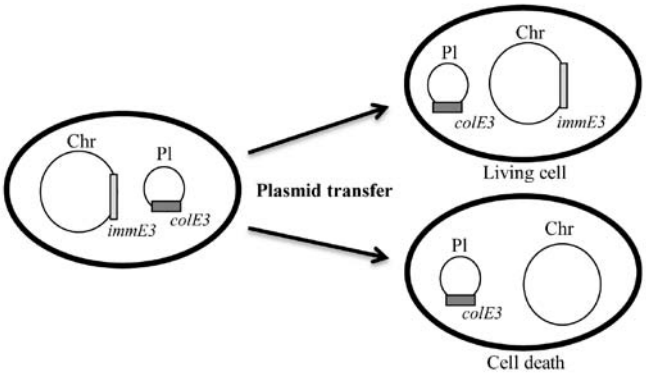

Fig. 2. Regulation of lethal gene. Horizontal transfer of the plasmid with *colE3* gene to another cell without *immE3* gene in bacterial chromosome leads to its death [20; modified]. Abbreviations: Chr – bacterial chromosome, Pl – plasmid. Explanation in text

GMMs for bioremediation purposes

The fusion of traditional microbiology, biochemistry, ecology and genetic engineering is a very promising solution for *in situ* bioremediation. Many reports showed that GMMs had higher predisposition to decay of various organic pollutants in comparison with natural strains [5]. The examples of selected GMMs degrading toxic organic compounds are presented in Table 1.

Table 1

GMMs degrading organic compounds

GMMs	Introduced gene/s	Organic compound	Reference
Escherichia coli AtzA	atrazine chlorohydrolase	atrazine	[34]
Pseudomonas fluorescens HK44	*luxCDABE*	naphthalene	[33]
Burkholderia cepacia L.S.2.4	pTOD plasmid	toluene	[2]
Pseudomonas fluorescens F113rifpcb*rrn*BP1::*gfp*-mut3	operon *bph*, *gfp*	chlorinated biphenyls	[4]
Pseudomonas putida KT2442(pNF142::Tn*Mo-d*-OTc)	pNF142 plasmid, *gfp*	naphthalene	[12]
Burkholderia cepacia VM1468	pTOM-Bu61 plasmid	toluene	[35]
Rhodococcus sp. RHA1(pRHD34::*fcb*)	*fcbABC* operon	2(4)-chlorobenzoate 2(4)-chlorobiphenyl	[32]
Pseudomonas putida PaW85	pWW0 plasmid	petroleum	[17]
Comamonas testosteroni SB3	pNB2::*dsRed* plasmid	3-chloroaniline	[3]
Escherichia coli JM109 (pGEX-AZR)	azoreductase gene	decolorize azo dyes, C.I. Direct Blue 71	[16]
Pseudomonas putida PaW340(pDH5)	pDH5 plasmid	4-chlorobenzoic acid	[22]

There is a wide range of possibilities for genetic manipulations of bacteria for bioremediation purposes. They include modification of enzyme specificity, designing of a new metabolic pathway and its regulation, introduction of marker gene for identification of recombinant in contaminated environment and construction of biosensor for detection of specific chemical compounds [9, 20, 33].

The genetic engineered *Pseudomonas fluorescens* HK44 was the first strain used in the field experiment. The aim of this study was to evaluate its applicability in long-term bioremediation of naphthalene contaminated soil and to visualize inoculated cells by bioluminescence image. *P. fluorescens* HK44 contained pUTK21 plasmid, which was made by transposon Tn*4431* insertion into NAH7 plasmid from *P. fluorescens* 5R. This transposon originated from *Vibrio fischeri* and carried *luxCDABE* gene cassette. The genes responsible for naphthalene degradation pathway and *lux* gene cassette were arranged under a common promoter what resulted in simultaneous degradation of naphthalene and luminescent signal [33]. The other genetically modified strain *P. putida* KT2442 (pNF142::Tn*Mod*-OTc) able to degrade naphthalene in soil was constructed by Filonov et al. [12]. For its construction three strains of bacteria were used. They included *Escherichia coli* S17-1 with pTn*Mod*-OTc plasmid (carrying tetracycline resistance gene), *Pseudomonas* sp. 142NF (pNF142) able to degrade naphthalene and *P. putida* KT2442 with *gfp* gene localized in chromosome. The results from this study confirmed that recombinant bacteria could degrade naphthalene and transfer pNF142::Tn*Mod*-OTc plasmid to autochthonous microorganisms.

The possibility of plasmid pWW0 transfer from *Pseudomonas putida* PaW85 capable of degrading petroleum hydrocarbons into rhizosphere bacteria was studied by Jussila et. al. [17]. They stated that horizontal gene transfer events in petroleum contaminated soil have occurred between PaW85 and *Pseudomonas oryzihabitans* 29. Due to bacteria colonizing rhizosphere could degrade petroleum-derived contaminants. In other study, Lipthay et al. [21] investigated the degradation of 2,4-dichlorofenoxyacetic acid (2,4-D) by bacteria *Ralstonia eutropha* and *Escherichia coli* HB101 carrying pRO103 plasmid. The plasmid contained gene encoding 2,4-dichlorophenoxyacetic acid/2-oksoglutaric dioxygenase. It was confirmed that obtained transconiugant *R. eutropha* (pRO103) significantly increased degradation of 2,4-D in soil.

Enzymatic breakdown of polychlorinated biphenyls (PCBs) by wild strains of bacteria leads to the formation of chlorobenzoic acid (CBA), which is toxic and may inhibit PCB biodegradation in soil. Rodrigues et al. [32] studied the ability of two genetically modified strains *Rhodococcus* sp. RHA1 (pRHD34::*fcb*) and *Burkholderia xenovorans* LB400 (pRO41) to degrade mixture of PCBs in soil polluted with Aroclor 1242. The wild *Rhodococcus* sp. RHA1 strain was equipped with *fcbABC* operon from *Arthrobacter globiformis* sp. KZT1. These genes were introduced into natural pRT1 plasmid from *Pyrococcus* sp. JT1 giving artificial PRHD34::*fcb* vector. In turn, LB400 (*ohb*) strain contained *ohbABCR* operon (from *Pseudomonas aeruginosa* 142) encoding enzymes responsible for *orto*-dehalogenation of mono-, di- and trichlorobenzoates. The *ohbABCR* gene cassette was inserted into pRT1 plasmid resulting recombinant pRO41 plasmid, which next was transferred into LB400 cells. The expression of introduced genes in LB400 (*ohb*) prevented the accumulation of 2-CBA and 2,4-CBA in soil inoculated with recombinant strain. The obtained results have also showed that the efficiency of Aroclor 1242 degradation in soil was not dependent on the number of inoculated recombinants RHA1 (pRHD34::*fcb*) and LB400 (pRO41).

4-chlorobenzoic acid (4-CBA) is a major stable intermediate in degradation of chloroaromatic compounds, especially PCB and *p,p'*-dichlorodiphenyltrichloroethane (DDT). Massa et al. [22] constructed *Pseudomonas putida* PaW340 (pDH5) strain, which was able to degrade 4-CBA in soil. Recombinant strain was obtained by cloning *fcb* genes encoding dehalogenase into artificial plasmid pDH5, which was introduced into *P. putida* PaW340 cells, non-growing in the presence of 4-CBA. The *fcb* genes responsible for hydrolytic dehalogenation of 4-CBA to 4-hydroxybenzoic acid (4-HBA) originated from donor *Arthrobacter* sp. FG1 strain. The obtained recombinant as well as donor of *fcb* genes were able to degrade 4-CBA effectively both in sterile and non-sterile soil. Therefore, they could be used in bioremediation of areas contaminated with 4-CBA.

Genetically modified microorganisms can be applied not only in degradation of toxic compounds but also in promotion of plant growth. Generally, plant growth-promoting bacteria (PGPB) are not able to stimulate plant growth in the presence of various toxic compounds [5, 9, 30]. For this reason, Yang et al. [41] tried to design genetically modified bacteria that could promote maize growth and degrade phenol simultaneously. Strains used for construction of such recombinant included phenol-degrading *Pseudomonas aeruginosa* SZH16 that was not able to promote plant growth and PGPB *Pseudomonas fluorescens* without ability to degrade phenol. As a result of horizontal gene transfer they obtained recombinant P13, which stimulated maize growth and degraded phenol effectively. In other study, Barac et al. [2] used genetic manipulation to improve the efficiency of toluene detoxification. For this purpose, they transferred toluene-degradation plasmid pTOD from donor *Burkholderia cepacia* G4 into natural endophyte strain of yellow lupine *B. cepacia* L.S.2.4. The obtained results showed that recombinant bacteria had potential for toluene degradation and reduced transpiration through the leaves in the range of 50-70%. Another natural host yellow lupine *B. cepacia* VM1468 was used by Taghavi et al. [35] in toluene degradation experiment. This endophyte strain was constructed by pTOM-Bu61 plasmid transfer from *B. cepacia* BU61 *via* conjugation into *B. cepacia* BU0072. It was confirmed that in the presence of engineered endophyte toluene transpiration through the aerial parts of the plants was 5-times lover than in control plants. Moreover, the increase about 30% of roots and

leafs mass was observed. These results indicated that plasmid pTOM-Bu61 could transfer naturally to other natural endophytes *in planta* and stimulate toluene degradation. The construction of naphthalene-degrading endophytic bacteria *Pseudomonas putida* VM1441 (pNAH7) was described by Germaine et al. [13]. They reported that endophytic strain could protect pea plants from some of the toxic effects of naphthalene. Moreover, inoculation of plants with recombinant resulted in higher about 40% efficacy of naphthalene removal in comparison with un-inoculated control plants.

The law regulations

The Polish law principles define genetically modified organisms in The Official Act on GMO 2001.76.811 of 25th July 2001 (article 3, statute of 22nd June 2001). In turn, decree of Minister of Justice of 8th July 2002 contains regulations on GMO, their use and release into environment. The Republic of Poland as a member of the European Union is also dependent of EU law. Basic regulations on GMO are contained in European Parliament and Council of Europe directives: 2001/204/WE of 8th March 2001, 2001/18/WE of 12th March,2001 and 2009/41/WE of 6th May 2009.

Conclusions

Biodegradation of toxic organic compounds in soil is a complex and multistage process. It proceeds effectively only in favourable environmental conditions. The efficacy of biodegradation depends not only on chemical structure of contaminants, soil structure, but also catabolic potential of microorganisms. Genetic engineering offers a great opportunity for the use of natural ability of bacteria in construction of GMMs. Unfortunately, they are applicable mainly in laboratory conditions. The new approach connected with the use plant-associated endophytic bacteria seems to be a very promising solution in remediation of contaminated areas. However, this field of study requires still much work in laboratory scale.

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The Lipids and Membrane Biophysics: Faraday Discussion 161

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