## *Chapter 16*

# Overarching issues in the environmental risk assessment of deliberate release of transgenic micro-organisms

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*A lot of work has been done on a large number of bacterial species that we know are present in the environment. This work has yielded important results for fundamental science as well as for biotechnological applications. But the environment has much more to offer in terms of bacterial variety, genomic variety and useful genes that remain to be discovered. One way to exploit these possibilities is the study of the soil metagenome, DNA sequences directly isolated from soil samples.*

*The genes that are isolated by the various techniques can be used in genetic engineering to improve bacterial strains that are available and that can be handled. This raises questions about risks, for instance the risk of horizontal transfer of these transgenes between organisms, i.e. between higher organisms and bacteria, as well as between different bacteria. One way to minimise the chances of such horizontal gene transfer (HGT) is to reduce the homology between transgenic DNA in donor organisms and the DNA in recipient organisms. With all the enthusiasm about the environment as a source of biodiversity, it should be recognised that the environment is very promising, but also extremely difficult to investigate, and difficult to control.*

## Exploitation of bacterial diversity in the environment

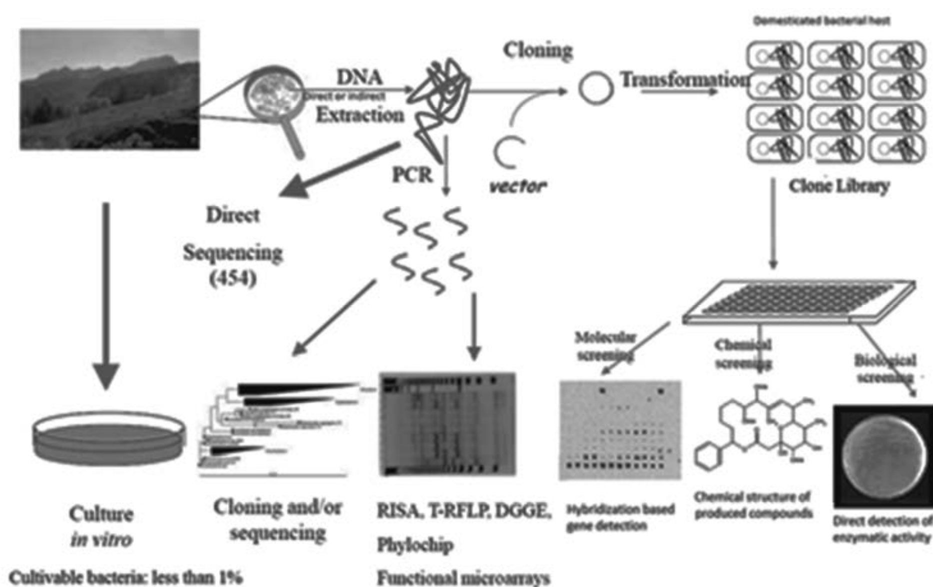
This volume has presented much fascinating information about prokaryotes in the environment and their (potential) roles in environmental processes. Still, it should be recognised that we have seen only a small part of what the environment has to offer in terms of useful micro-organisms and their functions. The list of organisms that are routinely used is already quite long, and the use of biotechnology and genetic modification may add organisms to the list, for instance attenuated strains of pathogens.

But there is a huge reservoir of microbial functions that has as yet been hardly tapped. But this is within reach: the functions that can be identified through metagenomics and exploited through genetic engineering are increasing rapidly, which together brings us the potential of "synthetic biology".

Metagenomics is the study of genes isolated directly from environmental samples, not from organisms cultured from environmental samples.

Figure 16.1 shows different ways to exploit the metagenome. Traditional experiments that look for cultivable bacteria will yield less than 1% of the existing diversity. By DNA extraction, the full genomic diversity can, in principle, be accessed (that is, if all DNA is extracted, see below). The DNA sequences can be analysed directly by various sequencing and hybridisation techniques or they can be cloned as a library, and the clones can be characterised by hybridisation-based gene detection, by analysing the chemical structure of produced compounds, or by direct detection of enzymatic activity.

Figure 16.1. **Metagenome exploitation**




*Source:* Lombard, N., et al. (2006) "La métagénomique des communautés microbiennes : Écologie microbienne des sols", *Biofutur (Puteaux)*, No. 268, pp. 24-27.

The method of hybridisation-based gene detection can be used to explore an environmental DNA sample for genes encoding a certain type of enzyme; for example the industrially interesting Type I polyketide synthases (PKSI). Using the ketosynthase domain of one of the enzymes, 140 of 60 000 clones showed up positive in hybridisation,

and 40 genes that were sequenced were all new PKSI type enzymes showing no redundancy (Ginolhac et al., 2004).

The concepts developed in metagenomics and the results obtained in practice are important for fundamental as well as for applied science. As we learn more about the genomes of a bacterial species, the species concept in bacteria becomes even more challenged than it was already. This has led to the development of the concept of the pangenome: the total genetic information that is found in all different strains that belong to a species. Thus, the isolation of new genes and new pathways is important for fundamental science, trying to explain the taxonomy of bacteria, as well as for biotechnology, where these new genes and pathways can be used and “new” organisms can be constructed using the newly characterised genes.

There is also an important issue for risk assessment. The fate of the new organisms with new genes after their inoculation into the environment should be known, as well as the fate of their genes that can be transferred from one organism to another by HGT.

## Horizontal gene transfer of transgenic DNA

The HGT of transgenic DNA, if and when it occurs, could be seen as biological pollution, and, in contrast to chemical pollution that is diluted over time, this biological pollution could be augmented in the environment if the new genes increase the fitness of the organisms. It should be kept in mind that a genetically engineered micro-organism, once it is released into the environment, cannot be called back.

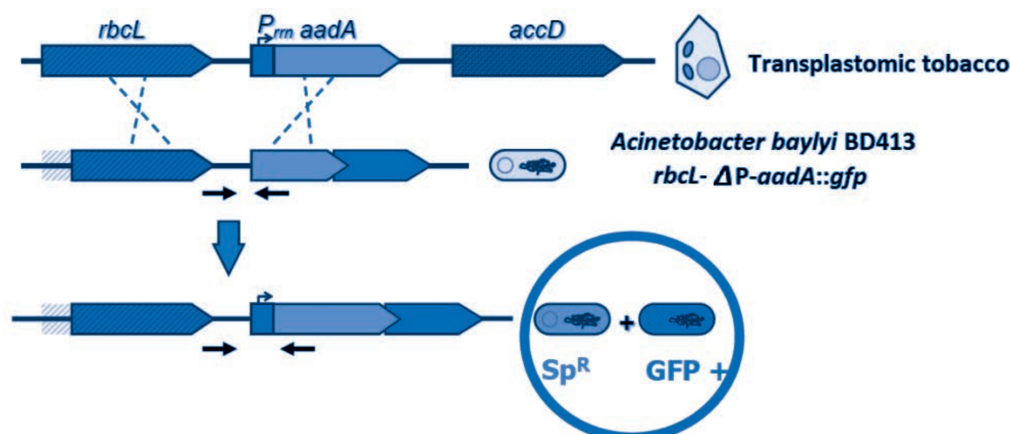
HGT is a very fundamental adaptive mechanism for prokaryotes. This is also evident from the sequences of bacterial genomes that are now known, and that all show evidence of being mosaic, i.e. made up of parts of genomes of other organisms. The question then becomes: what circumstances optimise HGT, and are there hot spots for HGT? A lot is known about the HGT of transgenic DNA from plant genomes to bacterial genomes, and from that we can learn some general facts.

First, Gebhard and Smalla (1998) showed that plant DNA carrying suitably selectable genes such as antibiotic resistance can be acquired by HGT *in vitro*. Transformation of bacteria also occurs *in situ*, as has been shown by Kay et al. (2002), who showed that in *Ralstonia*-infected plants there can be HGT of plant DNA to the *Acinetobacter baylyi* strain BD413. This gene transfer requires homology between the plant DNA and the bacterial DNA, but then the rate of gene transfer can be much higher in the plant than it is *in vitro*. HGT can now be visualised *in planta* (Figure 16.2; Pontiroli et al., 2009). Again, HGT is dependent on homology between the transforming DNA and resident DNA in the recipient.

Figure 16.3 shows transformants that result from HGT *in planta*, using this system.

The results show that sequence homology is the only barrier for HGT: when the experiment is set up in a way that there is sufficient sequence homology between the plant DNA and the DNA of the recipient micro-organism, HGT may occur at frequencies that can easily be observed, especially in decaying plant material. In conclusion: the potential for HGT in plant-bacteria interactions, and consequently also in interactions between bacteria, exists, and is an issue that has to be taken into account in risk assessment, but it can be mitigated by minimising sequence homology between transgenic DNA sequences and DNA sequences in potential recipients.

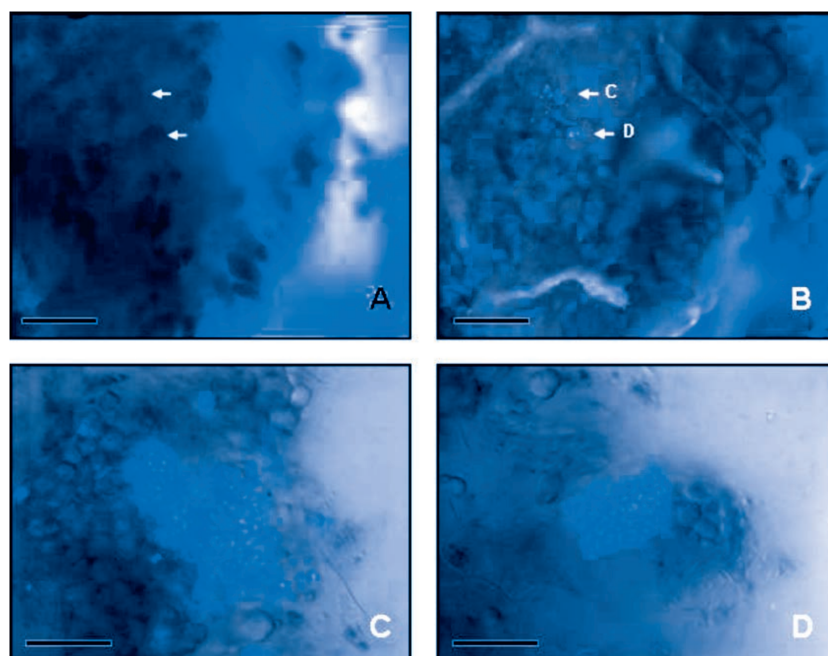
Figure 16.2. **Visualisation of horizontal gene transfer *in planta*: Genetic approach**




*Notes:* Transgenic chloroplast DNA carrying a bacterial *aadA* gene next to the chloroplast *sbcL* gene in the plant may transform *Acinetobacter baylyi* carrying the same genes, the *aadA* gene being deleted for the promoter and fused to a functional GFP gene. Recombination between the two gene sequences will result in *Acinetobacter baylyi* that has become spectinomycin resistant by expression of the *aadA* gene, and fluorescent by expression of the GFP gene.

*Source:* Pontiroli, A., et al. (2009), "Visual evidence of horizontal gene transfer between plants and bacteria in the phytosphere of transplastomic tobacco", *Applied and Environmental Microbiology*, No. 75, pp. 3 314-3 322.

Figure 16.3. **Visualisation of horizontal gene transfer *in planta***




*Notes:* A) Bright-field image, arrows point at the localisation of transformants. B) Epifluorescens micrograph showing transformants (green), chloroplasts (red) and veins (cyan. C and D) Details showing cell clusters of *A. baylyi* transformants expressing the GFP after restoration of the promoter activity through horizontal gene transfer between the plant and the bacteria. Bars: A, B: 50 µm; C, D: 20 µm.

*Source:* Pontiroli, A., et al. (2009), "Visual evidence of horizontal gene transfer between plants and bacteria in the phytosphere of transplastomic tobacco", *Applied and Environmental Microbiology*, No. 75, pp. 3 314-3 322.

## Soil as a heterogeneous and complex environment

Ever since we have known about the vast amount of non-cultivatable bacteria in the soil, it is clear that soil systems are extremely heterogeneous and complex. It is a reservoir of genetic diversity but also a reservoir of problems. The Metasoil project,[1] which aims to establish the complete genome sequence of a soil sample, has shown a number of problems that have to be overcome. One problem and one of the impediments for establishing the metagenome of a soil sample is the difficulty to ensure that "all" DNA is extracted from the soil. There are quite a number of DNA extraction methods available, and it was found that the identity and the diversity of the DNA sequences isolated is very much dependent on the number of different extraction methods employed. As an example, using only one extraction method yielded only about 40% of all the sequences present on the Rothamsted soil phylochip, while by the use of 15 different methods on the same soil sample, 99% of the sequences were found. Thus, using two different methods on the same soil sample will yield two different populations of DNA sequences, and the degree of difference is similar to when DNA sequences derived from two different soils are compared.

This means that the metagenome approach is very promising, but also fraught with limitations as long as there is not better control over the DNA sequences that are extracted from the soil.

The take-home lesson of these considerations is: the soil is a most interesting environment, but it is extremely difficult to investigate, and difficult to control.

## Note

1. www.genomenviron.org/Projects/METASOIL.html.

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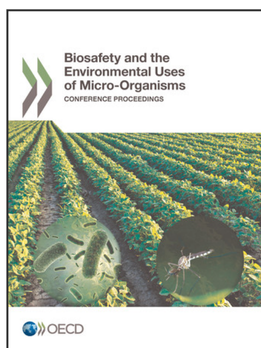


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