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SURVIVAL, PERSISTENCE, TRANSFER

An update on current knowledge on GMOs and the fate of their recombinant DNA

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Chapter 1

Introduction

During the last years the release, whether tolerated or permitted, of genetically modified microorganisms (GMOs) and their nucleic acids into various environments has increased worldwide. Legislation concerning the containment of GMOs has been deregulated and safety measures have been relaxed throughout the industrial world, as genetic engineering has not occasioned any obvious accident or visible negative impact during the two decades of its rapid development and constantly increasing use. Another development of the last 10 to 15 years, according to the 1996 World Health Organisation (WHO) Report, has been an increase in frequency of outbreaks of new and reemerging infectious diseases. Moreover, current pathogen strains are often resistant to known treatments, some even to nearly all commonly used antibiotics. Horizontal gene transfer is now recognised to be the main avenue of exchange of genetic material in the microbial world, and hence also of the exchange and spread of antibiotic resistance genes.

These developments give rise to two questions. Does the extensive use of antibiotic resistance genes in genetic engineering contribute to the increase in frequency of antibiotic resistance in bacterial pathogens? And what will be the outcome of a spread of recombinant genes analogous to the spread of antibiotic resistance genes?

Do we have risk assessment procedures available for monitoring the fate of GMOs and their recombinant DNA? Are we capable of

detecting ecological impacts or health impacts in early warning systems? The answer has to be no, and this for various reasons. We are only beginning to understand microbial ecology. There is a lack of basic knowledge by which to judge the possible impacts of a given GMO or its recombinant DNA on different environments. In particular, we do not know enough of the special conditions under which gene transfer takes place. What are the selective conditions which facilitate the transfer of specific genes and may, for example, promote the transfer of recombinant gene constructs?

A prerequisite for the possibility of GMOs and recombinant DNA contributing to the spread of newly cloned genes is the viability and/or persistence of GMOs and their recombinant DNA in certain environments. Another point requiring analysis is the extent to which artificial vectors facilitate and/or enhance the probability of horizontal gene transfer.

In this review we present the latest data on survival of GMOs in different environments, persistence of recombinant DNA, and what is currently known of the different gene transfer mechanisms involved.

Chapter 2

Survival and Spread of GMOs

Survival in the digestive tract

The digestive system of vertebrates and invertebrates alike offers possibilities for bacteria ingested with the food to get in close contact with each other and with the microflora of the animal. Its environment may prolong bacterial survival as compared with other environments and, due to its high density of microorganisms, increase the probability of gene transfer (Adamo and Gealt, 1996).

Moreover, bacteria can be disseminated by their new host animals, e.g. earthworms (Clegg et al., 1995). Some bacteria may even change their metabolic activities and capacity for survival during their passage through the digestive tract. (Clegg et al., 1995). Earthworm activities may also influence the rate of transfer of conjugative plasmids from *Pseudomonas fluorescens* to autochthonous soil microorganisms (Daane et al., 1996). It is also possible for transfer mechanisms other than conjugation to contribute to the dissemination of DNA from GMOs to the endogenous microflora of animals (Duval-Iflah, 1992).

Aside from these studies on bacterial interaction with the invertebrate digestive system, Brockmann et al. (1996) investigated the possibility of different strains of *Lactococcus lactis* colonising the rat intestine and the transfer of plasmids between these bacteria. While the digestive tract of germfree animals was found to be rapidly colonisable by all kinds of bacteria, this only succeeded temporar-

ily (for 2 - 3 days) in non-germfree controls. Studies with *Lactococcus lactis* in the human intestine yielded similar results (Klijn et al., 1995a). Moreover, the conjugative plasmid pAMß1, which has a broad host-range, proved transferable both to a *Lactococcus* recipient strain in the intestine of germ-free rats and an endogenous strain of *Enterococcus faecalis* in that of non-germfree animals (Brockmann et al., 1996). These authors conclude that even those microorganisms only temporarily detectable in the digestive tract are able to transfer their conjugative plasmids to the endogenous microflora. The rate of such events mainly depends on the structure of the plasmids themselves.

Survival in wastewater and sludge

The most important factor influencing bacterial survival in wastewater has to do with competitors. Because predator populations fluctuate seasonally, the time of year is an important factor in evaluating the risk of GMOs' survival (Inamori et al., 1992). If they are not eliminated by their competitors, *E. coli* K12 and *Pseudomonas putida* species have the best chances of survival when associated with snow particles containing sludge material (McClure et al., 1989; Overbeck, 1991). Whereas viable cell counts in supernatant were found to decrease quickly after introduction of recombinant *E. coli* K12 into the aeration basin of a model wastewater treatment plant, this was not usually the case in the remaining bacteria attached to sludge particles (Heitkamp et al., 1993).

Most studies on survival and gene transfer in wastewater and sludge use *in vitro* systems or models of settlement tanks or activated sludge for investigation. Ashelford et al. (1995) concentrated on percolating filter beds, which are layered with living cells (a so-called biofilm) similar to the river epilithon. Two inoculated *Pseudomonas* strains survived the whole investigation time of 145 days. The authors demonstrated the transfer of a conjugative plasmid (pQKH6) harboured by one of these strains to another,

initially plasmid-less, strain (Ashelford et al., 1995).

Similar results were also published by Feldmann and Sahm (1994), who investigated the survival of four different recombinant microorganisms in laboratory wastewater systems.[1] While none of the GMOs persisted in the settlement tank, two yeasts tested in the aeration basin remained detectable for more than 25 days.

Survival in aquatic ecosystems

A lot of the above data apply in a like manner to aquatic ecosystems. The chance of survival of, e.g., *E. coli* and *Campylobacter jejuni* increases in filtered water taken from lakes, since filtering eliminates predators and competitors (Korhonen and Martikainen, 1991). Sterile tapwater also allowed the persistence of four transgenic microorganisms tested within the scope of the "Verbundprojekt Sicherheitsforschung Gentechnik"[2]: in this medium survival lasted the whole testing period of 440 days (Tebbe et al., 1994a).

Brettar et al. (1994) collected data on the survival of *Pseudomonas putida* DSM3931 in lakewater mesocosms. Strains were not genetically modified and survived the whole testing period of 10 weeks. Mesocosms additionally inoculated with culture media even induced an increase of bacterial numbers during the first 10 days. The most important factor responsible for the reduction of bacterial populations was predation, though many bacteria escaped by associating with particles and/or sediments during the first two days (Brettar et al., 1994).

Predation through protozoa also eliminated most bacteria

[1] These experiments were part of the "Verbundprojekt Sicherheitsforschung Gentechnik", which aims at investigating the safety of GMOs released from production facilities, and were sponsored by Bayer AG, Germany.

[2] *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Corynebacterium glutamicum*, and *Hansenula polymorpha*.

(*Pseudomonas fluorescens* AG1) inoculated in seawater mesocosms, though the extent of this was found to depend on the state of bacterial growth (Christoffersen et al., 1995). Elimination of bacteria in seawater is also achieved by lysis through bacteriophages (bacterial viruses), depending on seasonal changes (Weinbauer et al., 1995).

Survival of bacteria in aquatic ecosystems also depends on abiotic factors like temperature, nutrient availability and osmotic conditions (Ahl et al., 1995). These authors studied the survival of *Pseudomonas fluorescens* Ag1 in experiments with seawater microcosms (0.5 l water) and seawater mesocosms (5,300 l water). Ahl et al. (1995) demonstrated a less pronounced reduction of bacterial cells inoculated in mesocosms as compared with earlier microcosmic experiments. Of the inoculated *Pseudomonads* 25% survived the whole testing period of 14 days.

Intensity and seasonal changes of light are further important abiotic factors influencing the survival of bacteria in natural environments (Canteras et al., 1995). Five different strains of *E. coli* kept in complete darkness persisted for 96 hours in sterile riverwater with only a minor reduction of cfu[3]. Differences in persistence were only apparent upon illumination. However, sterile conditions cannot be taken to represent natural aquatic ecosystems. In the comparative study of Alvarez et al. (1996b) on the survival of *E. coli* strains with or without plasmids (DH1 and JM103) in tropical riverwater systems, differences in survival only showed in unsterile test-chambers.

Bacteria taken from their natural environment, transformed with a plasmid and then released again as GMOs, have a very high chance of establishing themselves in their original ecosystem. For example, Sobecky et al. (1996) demonstrated that a marine bacterium (*Achromobacter* sp.) is able to form stable populations in seawater

[3] cfu = colony-forming units

microcosms within 2-3 weeks after being transformed with a plasmid bearing a gene encoding an alkaline phosphatase. Even the enzyme was proved to have retained its activity.

Bacteria not adapted to environmental conditions, like typical enteropathogenic microorganisms (e.g. *E. coli* ETEC, *Yersinia enterocolitica* and *Campylobacter jejuni*) are not only able to survive within their hosts at body temperature but may also persist in a cultivable stage for weeks in aquatic environments at temperatures between 6°C and 16°C (Terzieva and McFeters, 1991). Davies et al. (1995) demonstrated that the time of survival of enteropathogenic bacteria (faecal *Streptococci* and Coliforme) in sediments exceeds that in open water. While gram-negative bacteria inoculated in open seawater soon entered a stage of non-culturability, *E. coli* cells residing in sediments remained cultivable during the whole testing time of 68 days (Davies et al., 1995).

The same seems to be true of groundwater aquifers and freshwater environments, where sediments provide a better chance of survival than open water. This was demonstrated by Winkler et al. (1995) with *Burkholderia cepacia* in a groundwater microcosm experiment and by Fish and Pettibone (1995) with *E. coli*. The latter species remained cultivable in sediments for 56 days, i.e., for the entire duration of the test.

Survival in soil

Since soil environments are especially complex and heterogeneous systems, it is rather difficult to predict the chance of survival they offer to newly introduced GMOs. **The density of soil bacteria is estimated at about 108 to 109 cfu/g soil. However, less than 10% of these are cultivable using current techniques, and only around 1% have been characterised to date (Hugenholz and Pace, 1996; Pace, 1997).** In clay sediments bacteria are detectable at depths down to 224 m (Boivin-Jahns et al., 1996). Microbial populations from the laboratory decrease drastically during the first days after

their introduction into natural soil, but then often remain at a constant level (Schmidt, 1991).

It is most important in assessing and comparing published data on the survival of GMOs in soil systems to thoroughly analyse the design of the microcosm or mesocosm and detection method being used. The variety of microcosms studied makes it is rather difficult to directly compare the results collected (Angle et al., 1995). An undisturbed soil texture is an important prerequisite for optimal imitation of natural environmental conditions, as Angle et al. (1995) demonstrated. These authors compared survival of *Pseudomonas aureofaciens* in microcosms and deliberate release experiments in naked soil for two years. Validation of the microcosms used is also expedient with aquatic systems. Leser (1995) found 63%-76% agreement between data obtained from a lakewater microcosm and a natural lake. After inoculation of *Alcaligenes eutrophus* in both systems the degree of coincidence was reduced to 2-27%, but increased again with decreasing cell counts (Leser, 1995).

As with aquatic and wastewater systems, in soils, too, predation through protozoa is one of the most important factors influencing the persistence of inoculated GMOs. In these environments protozoal activity requires a certain degree of soil moisture and free water in soil pores of suitable size (Van Elsas 1992; Wright et al., 1995). Experiments in soil microcosms with *Pseudomonas fluorescens* and the ciliate *Colopoda steinii* demonstrated that small pores (< 6µm) may offer effective protection zones against predators (Wright et al., 1995). While bacterial populations in larger pores (< 30µm) diminish more rapidly, bacteria that do survive show a higher level of metabolic activity in response to the richer supply of nutrients there (Wright et al., 1995). Van Der Hoeven et al. (1996) used a computer simulation model to evaluate the chance of survival of GMOs in soil pores of different sizes. Bacteria introduced into the soil were eliminated very slowly, in some cases taking years for total elimination (Van Der Hoevan et al., 1996).

The number of bacteria inoculated into the soil is a very important determinant of the time of possible survival, as differences as small as 100 *E. coli* K12 cells/g soil suffice to change the results of an assay (Recorbet et al., 1992). This strong correlation between inoculation number and survival is also found in aquatic ecosystems (Karapinar and Sahika, 1991). Another criterion is the depth of inoculation. In the range from 10 to 50 cm the chance of survival of the phytopathogenic bacterium *Erwinia carotovora* increases with growing inoculation depth. This correlation largely reflects differences in competitive pressure (Armon et al., 1995). Recorbet et al. (1995) demonstrated uneven distribution of genetically modified *E. coli* which had been inoculated in soil systems. Furthermore, GMOs were concentrated in different soil layers than autochthonous bacteria.

Spore-forming bacteria like *Bacillus subtilis* are particularly likely to persist for prolonged time periods. Spores of *Bacillus subtilis* of both native and genetically modified parent strains remained culturable in sterile soil for at least 50 days (Tokuda et al., 1995). GMOs' ability to adapt to natural environments makes it more difficult to estimate their chance of survival after release. Cells of *Pseudomonas fluorescens* showed improved stress resistance against temperature or osmotic pressure one day after inoculation into two different types of soil (Van Overbeek et al., 1995). When cultivated under starving conditions in the laboratory these bacteria may stand stress-factors like "hunger" for 70 days by reducing their cell size. These physiological changes are soon reverted again when conditions are made to improve (Clegg et al., 1996).

There is still no evidence of plasmid-bearing microorganisms being less fit than their native parents: e.g. Fujimura et al. (1994) tested genetically modified *Saccharomyces cerevisiae* and their parent strains under different environmental conditions but were unable to prove any negative impact in terms of survival probability. Under selective pressure, exerted, for example, by antibiotic substances, GMOs

may even profit from possessing the corresponding resistance genes (Van Elsas, 1992). This was also demonstrated by Ramos et al. (1994) with recombinant *Pseudomonas putida* (pWW0-EB62) in soil systems. In unsterile soil and under assumed selective pressure cells numbers even increased after inoculation (Ramos et al., 1994). However, application of selective pressure is not a necessary prerequisite for survival of GMOs.

Sjogren (1995) initiated a very profound project on survival of *E. coli* using a multiresistant native strain isolated from a lake. The bacteria were inoculated into different field plots seeded with rye. There were no more bacteria detectable on the rye grass 41 days later. After two months bacteria were reisolated from soil at a depth of 20 to 50 cm. At 60-cm depth they reached groundwater and persisted there at the water-soil interface for two more years. Six years later 1.5 cfu/ml were still detectable, and after eight years cell counts had increased to 8 cfu/ml. After 13 days, at the end of the experiment, population density was still at 0.25 cfu/ml (Sjogren, 1995). The author calculated the speed of distribution as about 2 cm/day. In analogous experiments performed under laboratory conditions with microcosms of the same soil the maximum survival time had been 2.5 years (Sjogren, 1995).

GMOs intended for use in natural environments are designed to persist and show at least some competitive ability versus autochthonous microorganisms. Experiments with *Rhizobium meliloti*, which had been genetically modified for biological containment by disrupting a gene for recombination, only demonstrated reduced survival of the genetically modified as compared with the native strain when performed in model ecosystems, but not after deliberate release in agricultural soils (Dresing et al., 1995; Selbitschka et al., 1994).

Beside surviving, GMOs inoculated in soil may also be distributed further, thus increasing the chances of gene transfer. Gillespie et al. (1995) simulated rainfall on wheat plants or naked soil in green-

house experiments after inoculation with genetically modified *Pseudomonas aureofaciens*. The number of cells detectable in the soil was found to correlate with the number of cells in water running from the plots. One exception to this was that 245 days after inoculation 108 cfu were measured in the water probes, whereas cell counts in the soil were below the detection limit. The authors concluded that standard techniques for detection of GMOs in soil may not be suitable for registering bacteria in all soil layers and that short and heavy rainfalls can cause quite high numbers of GMOs (about 1,014 cfu/ha) to be washed away from the place of release (Gillespie et al., 1995). Similar testing was done by Hekman et al. (1995) with *Pseudomonas fluorescens* and *Burkholderia cepacia*. In the case of planted microcosms, these authors demonstrated enhanced distribution of inoculated bacteria in deeper soil layers after moderate rainfall (Hekman et al., 1995).

Survival on plants

It is evident from the last paragraph that any analysis of survival of GMOs in soil has to include the plants growing there. A recombinant strain of *Bacillus amyloliquefaciens*, used to produce (-amylase) was no longer detectable in the soil of planted microcosms six days after inoculation, though it persisted on the grass for at least 70 days (Wendt-Potthoff et al., 1994). Genetically modified strains of the phytopathogenic bacteria *Xanthomonas campestris* inoculated on cabbage host plants survived for six months and were distributed to other cruciferous plants and even unrelated plants (Dane and Shaw, 1996).

Persistence of bacteria inoculated on plants may depend on the plant species used. The population density of two strains of *Pseudomonas fluorescens* remained at a constant level on leaf surfaces of capsicum and eggplants during a 30-day glasshouse experiment, but decreased on leaves of strawberry and tomato (Cirvilleri et al., 1996). Genetically modified *Pseudomonas fluorescens* whose parent strain had been isolated from sugar beets persisted

Chapter 3

Further Points to Consider: VNC state and adaptation ability

A clear distinction must be made between cultivable and dormant cells, regardless of the environment under study (Dott et al., 1991; Turpin et al., 1993). The dormancy problem is currently receiving more and more attention, since results have been found to differ tremendously according to whether all cells or only cultivable cells (Kragelund and Nybroe, 1996; Pickup, 1991; Stotzky et al., 1991) are counted.

These VNC, i.e., viable non-culturable, bacteria are predominant in marine environments with high salt concentration (Byrd and Colwell, 1990; Garcia-Lara et al., 1993). There are various especially adapted methods of detecting VNC cells and cultivable cells. However, counts of VNC bacteria in environmental probes are usually rather variable (Bianchi and Giuliano, 1996; Byrd and Colwell, 1990; Jensen et al., 1996; Zweifel and Hagström, 1995). The problem of poor culturability has also to be addressed in the case of bacteria in activated sludge or soil probes (Binnerup et al., 1993; Wagner et al., 1993).

VNC cells can be distinguished from dead cells by some metabolism and protein synthesis parameters. Moreover, rather than lose their plasmids, they may even enrich their intracellular plasmid content (Arturo-Schaan et al., 1996; Nwoguh et al., 1995; Nybroe et al., 1996). These so-called dormant cells can be activated again when environmental conditions change. This is not to say that an im-

proved nutritional state is the only factor responsible for reactivation; temperature fluctuations in natural environments may also play a role (Colwell et al., 1996; Ferguson et al., 1995; Jiang and Chai, 1996; Oliver et al., 1995a; Rahman et al., 1996). The presence of plasmids can further contribute to induction of the VNC stage (Oliver et al., 1995b). Bacteria detection methods which rely on the expression of marker genes and are thus blind to VNC cells should therefore at least be combined with techniques that record VNC cells (Lee et al., 1996; Leff and Leff, 1996; Oliver et al., 1995b).

Obviously, the choice of a detection method has a great influence on the record of bacteria released in natural environments. The ability of microorganisms to adsorb to particles and sediments renders detection more difficult, though application of PCR has certainly lowered detection limits (Bej et al., 1990; Hodson et al., 1995; Lindahl 1996; Lindahl and Bakken, 1995; Tsushima et al., 1995; Vahjen and Tebbe, 1994). One disadvantage of this technique is the remaining difficulty in distinguishing between isolated DNA and DNA associated with living or VNC organisms (Brockmann et al., 1996; England et al., 1995; Tamanai-Shacoori et al., 1996).

A further point to consider in the context of survival of GMOs in natural environments is their capability to adapt to unfavourable environmental conditions. When given a chance to adapt slowly, bacterial populations in aquatic microcosms imitating natural surroundings show prolonged persistence as compared to non-adapted populations of the same strain (Awong et al., 1990; Mezrioui et al., 1994; Sobecky et al., 1992). Bacteria cultivated in the laboratory while exposed to various primary stress factors like unfavourable pH or nutrient deficiency are able to adapt, and also show higher resistance against different secondary stress factors (Ferianc et al., 1995; Lou and Yousef, 1996). Consequently, any design of microcosm or mesocosm experiments should include at least some environmental stress factors in order to check GMOs' responsiveness to them. In determining the time-scale of such experiments one has to consider that adaptation may be a slow process and that populations

of GMOs no longer detectable after a short time may multiply and rise again (Clegg et al., 1995; Gillespie et al., 1995; Sjorgen, 1995; Ramos et al., 1994; Thompson et al., 1995b). Stress factors may even act as a positive selection pressure.

Chapter 4

Spread of Cloned Sequences

Risk assessment should not be satisfied with the assumption or proof that a given GMO will not survive. It must rather extend to the fate of the DNA, which may be stably integrated, eventually expressed and may by chance provide advantages for indigenous microorganisms (Henschke and Schmidt, 1990; Istock, 1991; Schmidt et al., 1994). GMOs may only require a short time of survival in order to be transported to other ecological niches or be able to transfer some of their nucleic acids to other members of a given ecosystem (Byzov et al., 1993; Heijnen and Marinissen, 1995; Weiskel et al., 1996). While conjugation and transduction are restricted to living cells, transformation may also take place after the death of donor cells. The probability of gene transfer rises with the stability and quantity of isolated DNA (Fulthorpe and Wyndham, 1991), though selective forces have also to be taken into account and may play a crucial role.

For example, Pukall et al. (1996) isolated plasmids with mobilising capability from soil probes and pig manure in numbers substantially higher than estimated. Such plasmids can transform competent cells living in soil or manure environments also under suboptimal conditions and may even be able to mobilise recombinant plasmids in these cells (Lorenz and Wackernagel, 1987; Pukall et al., 1996).

Persistence of "naked" DNA

Isolated DNA does not only originate from dead and lysed cells but can also be actively secreted (Paget and Simonet, 1994). Quantifying extracellular DNA in environmental media seems to pose difficulties, judging by the great variability of published data (Lorenz and Wackernagel, 1994). Some authors assume that bacteria in natural environments release large quantities of high molecular DNA and plasmids into the extracellular gene pool (Lorenz and Wackernagel, 1994; Romanowski et al., 1990; Wackernagel et al. 1992). Secretion of DNA and competence for nucleic acid uptake are induced in particular in unfavourable environmental conditions like starvation (Lorenz and Wackernagel, 1994; Wackernagel et al., 1992).

Persistence in wastewater treatment plants (water/sludge)

Wastewater treatment plants guarantee rapid and almost complete inactivation and degradation of isolated DNA when it is suspended in open water. Nucleic acids adsorbed to sludge particles, however, find some degree of protection against degradation and also against detection (Gross et al., 1994; Aardema et al., 1983; Bauda et al., 1995). As efficiency of inactivation of naked DNA depends on different factors like temperature, it varies with the season and type of treatment plant under study (Bergemann et al., 1994; Lorenz and Wackernagel, 1994).

Persistence in aquatic systems

Some recent studies have dealt with the origin and distribution of naked DNA in aquatic systems (Beebee 1993; Hermansson and Linberg, 1994; Jiang and Paul, 1995; Weinbauber et al., 1995). As in wastewater environments, isolated and dissolved nucleic acids are

rapidly degraded in aquatic systems by enzymatic activity (Alvarez et al., 1996a; Lorenz and Wackernagel, 1994). Adsorption to sand or clay minerals protects DNA against nucleases even if these are bound to the same particles (Aardema et al., 1983; Romanowski et al., 1993a).

Prolonged protection of nucleic acids against inactivation is also provided by VNC cells (Byrd et al., 1992; Lorenz and Wackernagel, 1994). If naked DNA remains biologically active, this can give rise to the transformation of competent cells. Therefore, transformation experiments are a suitable method of detecting intact molecules, regardless of whether they were adsorbed to particles or not (Alvarez et al., 1996a; Chamier et al., 1993; Mieschendahl and Danneberg, 1994; Romanowski et al., 1993a).

Persistence in soils

Isolated DNA in non-sterile soils can persist for some time, depending on various factors like temperature, pH, salt concentration, type of soil and nucleic acid material, all of which may influence the binding of DNA on mineral or quartz particles (Lorenz and Wackernagel, 1992). As in aquatic systems, when introduced into non-sterile soils, particle-adsorbed nucleic acids are protected against degradation by nucleases, even if these are bound to the same particle samples (Khanna and Stotzky, 1992; Lorenz and Wackernagel, 1987). Nucleic acids can bind to minerals quite rapidly and relatively independently of pH (Lorenz and Wackernagel, 1994). One factor determining the speed of association in aquatic systems is the structure of the DNA (Khanna and Stotzky 1992; Lorenz and Wackernagel, 1992; Lorenz et al., 1988; Paget et al., 1993). Factors relevant to soil environment include the humidity and granular structure of the soil (Hobom, 1995).

Data on the persistence of isolated DNA in soils show great variation. Smalla (1995) detected recombinant DNA from sugar beets in soil 18 months after inoculation of plant material. Romanowski et

al. (1993b) found that particle-associated plasmids could persist in different soils for at least 60 days. Plasmid conformation remained intact during the first two days in this study. According to Wackernagel et al. (1992) and Romanowski et al. (1992), the time-limit for detecting plasmids in unsterile soil probes without PCR application is five to ten days. Intact genes or plasmids can frequently be detected for as long as the originating bacteria.

Persistence in the digestive system

Consequently, if isolated DNA is able to persist in different environmental media, then it is quite possible for the digestive system of animals or humans to take up these nucleic acids with the food or drinking water. Moreover, as Schubbert et al. (1996) demonstrated in feeding experiments on mice, nucleic acids reaching the gastrointestinal tract must not necessarily be completely fragmented, and hence inactivated, but may just as well reach the bloodstream and temporarily even be detected in leukocytes and liver cells. The transferability of these results from mice experiments to other mammalian digestive systems, including the human one, is an obvious possibility. Investigating the persistence of bacterial cells (*Lactococcus lactis*) and their DNA in the human intestine Klijn et al. (1995b) were able to isolate the bacterial DNA from faeces four days after administering the inoculated milk drinks.

"Avenues and barriers of genetic transmission" (Istock, 1991)

To date gene transfer has mostly been found by means of specially designed plasmids, as in a number of studies carried out in microcosms (Collard et al., 1993; Del Solar et al., 1993; Mazodier and Davies 1991; Miller et al., 1992; Paget et al., 1992; Paul et al., 1991; Paul, 1992). However, the spread of antibiotic resistance markers throughout bacterial communities shows that gene transfer is likely to happen not only in more or less artificial settings but also under natural conditions (Saye and Miller 1989; Götz et al., 1996; Kruse

and Sorum, 1994). Moreover, some plasmids originating from gram-positive bacteria have also been isolated in gram-negative bacteria, stressing the possibility of a wide distribution of genetic information. It was not possible to ascertain in this case whether gene transfer took place via conjugation, transformation or transduction (Del Solar et al., 1993).

Conjugation, i.e., the transfer of nucleic acids by direct contact between bacterial donor and recipient cells, is assumed to be the most effective mechanism of genetic transmission under natural conditions. Wastewater treatment plants offer perfect conditions for this kind of transmission because of their abundance of cell populations (Schneider et al., 1994). Analogously, conjugation in the gastrointestinal tract is promoted by high population densities of potential bacterial donor and receptor cells (Morrison, 1996). Several recent studies have dealt with conjugation in digestive systems (Adamo and Gealt, 1996; Brockmann et al., 1996; Klijn et al., 1995a,b; Rang et al., 1996; Rybachenko et al., 1996). Microcosm experiments or *in situ* testing with soil or aquatic systems also demonstrate the transfer of plasmids by conjugation in other environments (Lebaron et al., 1994; Sandaa and Enger, 1994; Bale et al., 1988; Pukall et al., 1996; Lukin and Prozorov, 1992). Barkay et al. (1995) have pointed out the importance of using autochthonous bacteria as donors or recipients for efficient detection of transfer through conjugation in natural environments. Other authors have discussed the influence of different soil systems and supplements of plant material, temperature and soil humidity (Ashelford et al., 1995; Hermansson and Linberg, 1994; Klingmüller, 1993; Temann et al., 1992; Van Elsas et al., 1990). The main factors inhibiting or promoting conjugation in natural environments are thought to be selective forces from, e.g., heavy metals, herbicides, or antibiotics (De Rore et al., 1994a,b; Gadkari, 1991; Klingmüller and Rieder, 1994; Kozdroj and Pietrowska-Seget, 1995). Lafuente et al. (1996) have pointed out that conjugation in sterile soil microcosms is most efficient in "natural" abiotic conditions. As conjugation is an energy-consuming process, it can be promoted by any of a variety of

nutrients (Götz and Smalla, 1997). Any environmental compartment fulfilling this requirement like sediments, biofilms or water-air interfaces, and additionally characterised by high numbers of bacterial cells, will provide optimal conditions for conjugation (Amabile-Cuevas and Chicurel, 1996; Barkay et al., 1995; Hermansson and Linberg, 1994).

Transduction, a gene transmission process mediated by bacteriophages, requires living cells. It is more likely to occur under conditions of high metabolic activity (Ripp and Miller, 1995). Transduction may contribute to the spread of plasmids which are not transferable by conjugation for lack of essential sequences (Replicon et al., 1995). It is not restricted to plasmids, as chromosomal DNA can be transferred as well (Lorenz and Wackernagel, 1993). In this case the host range of the phages determines the possible radius of distribution of genetic material. Empirical evidence of gene transfer by transduction also taking place in natural environments (Ogunseitan et al., 1992; Ripp et al., 1994; Schicklmaier and Schmieger, 1995; Stotzky and Babich, 1994) has been further substantiated by experiments carried out under natural conditions (Kidambi et al., 1994; Miller et al., 1992) and by the detection of high numbers of phage particles in some environmental media (Kokjohn and Miller, 1992; Lorenz and Wackernagel, 1993; Schäfer, 1996). As transduction requires a certain minimum concentration of phage particles and corresponding bacterial host cells in order to be efficient, it is more likely to occur in environmental compartments characterised by high cell density and plenteous nutrients (Ogunseitan et al., 1992; Replicon et al., 1995). This is not an unconditional prerequisite for transduction, however, since also oligothrophic conditions and natural cell densities allow transduction (Goodman, 1994; Ripp et al., 1994). Adsorption of phages and bacteria to particles further increases the chances of survival and interaction and, consequently, also of transduction (Pickup et al., 1993; Ripp and Miller, 1995).

Transformation, the third mechanism of gene transfer, does not

depend on living cells as donors of nucleic acids. It requires the active uptake of isolated DNA by recipient cells which are in a physiological stage of competence (Lorenz and Wackernagel, 1994). According to present knowledge, induction of competence is determined by growth stage, impact of stress factors and/or presence of diffusible competence factors secreted by bacteria, and, not least, by the species in question (Baur et al., 1996; Cheng et al., 1997; Goodman, 1994; Lorenz and Wackernagel, 1994; Prozorov, 1997; Schlüter and Potrykus, 1996). The next step towards establishment of the foreign nucleic acids involves recombination as a means of integrating the new sequences into the recipient genome. Homologous sequences have better chances of transformation, though uptake does not usually depend on the presence of similar DNA (Lorenz and Wackernagel, 1994; Prozorov, 1997).

Establishment of plasmids does not depend on homology, **but only on suitable origins of replication** (Lorenz and Wackernagel, 1994). Experimental and empirical data both suggest that transformation is common in natural environments (Boyle-Vavra and Seifert, 1996; Hermansson and Linberg, 1994; Lorenz and Wackernagel, 1994; Schäfer, 1996). Microorganisms in aquatic and soil systems can also be transformed by isolated DNA, as has been demonstrated by Frischer et al. (1994), Lorenz and Wackernagel (1994), Paget and Simonet (1993) and others. Transformation of endogenous bacteria in the gastrointestinal tract was shown in the intestine of *Folsomia candida* after feeding with GMOs (Hobom 1995; Tebbe et al., 1994b). Baur et al. (1996) postulate that natural environments can provide optimal conditions for transformation which are not properly imitable in the laboratory. Environmental factors influencing transformation include lack or oversupply of nutrients and certain minerals, ionic strength of water and temperature (Baur et al., 1996; Frischer et al., 1993; Hermansson and Linberg, 1994; Lunsford and London, 1996; Williams et al., 1996). As in the case of conjugation and transduction, enhanced rates of transformation are found on surfaces and biofilms (Baur et al., 1996; Hermansson and Linberg, 1994).

Reports of intense natural gene transfer may appear to stand in contradiction to the ability of bacterial strains and varieties to maintain their genetic make-up. However, there are restriction systems whose action consists much rather in degrading or "silencing" foreign DNA than in preventing uptake of foreign DNA (Heinemann, 1991). Plasmid incompatibility is another means of hindering genetic transfer (Naik et al., 1994). Stressful conditions seem to reduce the activity of restriction systems in bacteria and to induce genes mediating recombination (Saunders and Saunders, 1993; Schäfer et al., 1994).

On the one hand, special plasmids are being designed to reinforce the constraints of biological containment (safety vectors without transfer genes), while on the other, progress in cloning relies on shuttle vectors capable of transgressing existing borders between bacterial classes and even kingdoms (Doucet-Populaire, 1992; Henchke and Schmidt 1990; Schäfer et al., 1994; Trieu-Cuot et al., 1987). Plasmids are being constructed to undermine the different levels of restriction they encounter under normal conditions in a natural environment and so ensure their persistence and integration (Dunn et al., 1993; Heinemann, 1991).

Chapter 5

Discussion

Current security measures for research and production are based on earlier assumptions about the survival and transfer abilities of microorganisms. In our opinion, the data in this article expose the concept of biological containment, on which legal regulation is based, as unsound, and this for several reasons:

- GMOs can survive or transfer their transgenes to indigenous organisms;

- DNA is more stable than has been hitherto imagined;

- and DNA taken up with the food is not completely degraded in the gastrointestinal tract, but has rather been found to enter white blood cells and spleen and liver cells.

- DNA can even be transferred to the cells of foetuses, as has been shown in newborn mice. Here transfer probably took place via the placenta (Doerfler and Schubbert, 1997).

Moreover, there are now nucleic acid constructs containing sequences which contribute not only to effective replication in different cellular backgrounds but also to stability and integration via recombination, transfer and extraordinary expression. They are — this is the essence of the art — especially designed to fulfil these jobs.

Moreover, neither laboratory nor *in situ* studies on survival, DNA persistence and gene transfer provide a realistic measure of what can happen in complex terrestrial, aquatic and gut environments (Spielman et al., 1996; Stotzky et al., 1991). Crude as they may be, validated microcosm and mesocosm studies imitating natural conditions as accurately as possible are nevertheless an indispensable means of testing and prognosticating the influence of some of the biotic and abiotic factors operating on GMOs in natural environments (Pickup et al., 1993; Spielman et al., 1996).

What has been neglected until now in designing such studies are the selective forces which may act on the spread of recombinant genes. Especially in disturbed or polluted environments, contaminants like heavy metals or high salt concentrations may possibly facilitate gene transfer.

In a recent seminar organised by the Norwegian Biotechnology Advisory Board on Antibiotic Resistance, Marker Genes and Transgenic Plants one of the invited speakers summarised:

"The extent and consequences of horizontal gene transfer are apparent in the evolution of antibiotic resistant microorganisms and evidence suggests that horizontal gene transfer may be equally frequent among multicellular eukaryotic organisms. But the actual and potential frequencies of gene transfer are poor indicators of risk; very common genes are not maintained in nature if unselected and rare genes become common extremely quickly if they are the subject of selection. What remains essential to assessing risk is identifying all potential selective pressures a recombinant gene might be suited to neutralise. New evidence suggests that current knowledge of evolutionary theory is inadequate to predict the fate of recombinant organisms or recombinant genes." (Heinemann, 1997).

To this we would add that until there is sufficient scientific evidence that a given GMO or its recombinant genes will not pose

any environmental stress or health impact, we should abide by the precautionary approach in regulating the contained use and deliberate release of GMOs. This implies putting a stop to deregulation in favour of contained use and to tolerated releases from production plants where environmental impacts are not routinely assessed. We should bear in mind that soil ecology, for example, has much to do with mineralisation and nutrient flow, which again is dependent on the enzymatic properties of the organismal network. Genes currently perceived as harmless like many enzyme-coding genes (or the organisms containing them) may alter soil chemistry and so create selective pressure or conditions favourable to the survival of GMOs (see Holmes, 1995 and for a review Doyle et al., 1995).

Moreover, there is no way to extrapolate from one region or environment to another, differing, environment. This is especially true when GMOs are transferred to ecosystems and climates which differ greatly from those where they were first developed and used. This is evident and acknowledged for deliberate release but also holds true for contained use in production plants with their multiple pathways for escape. Therefore, the scope of the biosafety protocol should cover the transfer of GMOs intended for contained use and have these included in the Advanced Informed Agreement procedures.

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