Horizontal transfer of antibiotic resistance genes from transgenic plants to bacteria - are there new data to fuel the debate?

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Dedicated to Professor Rudolf Casper on the occasion of his 70th birthday

Several thousand field releases of genetically modified plants (GMP) have been performed during the last decade. Presently, the majority of the GMP tested in the field or already commercialized contain bacterial antibiotic resistance genes. The antibiotic resistance genes are often of importance to select for transformants. However, in some transgenic plants the antibiotic resistance genes under the control of bacterial promotors were not used for the selection of transformants but are remains of the vector construct used for transformation. In any case, the antibiotic resistance genes do not encode desirable traits in commercial plants. Nevertheless the presence of antibiotic resistance genes such as the *npt*II gene in transgenic plants seemed to be accepted by competent authorities and by the public, this largely based on the fact that antibiotic resistance genes used as markers in transgenic plants were already abundant in environmental bacteria (Smalla et al., 1993), and that bacteria possess highly efficient means such as transferable plasmids or conjugative transposons to exchange genes between bacteria belonging to the same species, or between a wide range of bacterial hosts (Tschäpe, 1994; Davies, 1994, 1997). Furthermore, so far there was no experimental evidence that horizontal gene transfer (HGT) of genetic material from plants to bacteria can occur at all (Becker et al., 1994; Broer et al., 1996; Schlüter et al., 1995; Nielsen et al., 1997c).
During the last few years the present crisis of antibiotics became more and more evident due to the emergence of bacteria which are resistant to multiple, and sometimes even to all antibiotics (Levy, 1997; Amtsblatt der Europäischen Gemeinschaft, 1998). This has led to a new discussion about the hypothetic acquisition of plant-harbored antibiotic resistance genes from transgenic plants by bacterial communities and the potential impact of such a transfer event. Is it the public health perception that changed, or are there new data on HGT from plants to bacteria that fuel the debate among scientists and in the public?
Here we briefly summarize recent research results on the persistence of transgenic plant DNA in soil and horizontal transfer of antibiotic resistance genes from plants to bacteria. These findings mostly obtained under optimized laboratory conditions must be seen in context with the prevalence of the respective antibiotic resistance genes in environmental bacteria, the clinical importance of the respective antibiotics, and, most importantly, the selective pressure by the medical and agricultural use of respective antibiotics.

Natural transformation, the most likely mechanism for horizontal transfer of antibiotic resistance genes from transgenic crops to bacteria

The mechanism which most likely contributes to a horizontal transfer of antibiotic resistance genes from transgenic plants to bacteria is termed "natural transformation" (Nielsen et al., 1998, Bertolla and Simonet, 1999). Natural transformation is defined as a DNase sensitive process by which competent bacteria can take up free DNA (Stuart and Carlson, 1986). The single-stranded DNA taken up by the bacteria can either be stably integrated into the bacterial genome by homologous recombination, or form an autonomous replicating element. Other processes which could lead to stable replication are illegitimate recombination or the integration into integrons, e.g., if the antibiotic resistance gene is a gene cassette such as the *aad*A gene. Natural transformation provides a mechanism of gene transfer that enables competent bacteria to generate genetic variability by "sampling" of DNA present in their surroundings (Nielsen et al., 2000a). From laboratory experiments, more than 40 bacterial species from different environments are known to be naturally transformable (Lorenz and Wackernagel, 1994; Nielsen et al., 1998). Only a few cases of interspecies transfer of chromosomal genes between environmental isolates have been demonstrated to occur by natural transformation. Prerequisites for natural transformation are the availability of free DNA, the development of competence, the take-up and stable integration of the captured DNA. However, there is very limited knowledge of how

important natural transformation by bacterial or plant DNA is in different environmental settings such as soil, compost, manure, sewage, etc. Two aspects have been, or are presently studied, by several groups to address natural transformation in the environment:

- How long does free DNA persist, e.g. in soil, and is the DNA still available for natural transformation?
- How frequently do different bacterial species under environmental conditions reach a state of being able to take up free DNA?

Persistence of free DNA in soil

Investigations made by different groups have shown that in spite of the ubiquitous occurrence of DNases high-molecular free DNA could be detected in different environments. Free DNA released from microorganisms or decaying plant material can serve as a nutrient source or as a reservoir of genetic information for autochthonous bacteria. Different abiotic and biotic factors seem to affect the persistence of free DNA in soil. The content and type of clay minerals influence the extent to which free DNA is adsorbed to mineral surfaces and, thus, is protected from degradation by nucleases (Ogram et al., 1988; Romanowski et al., 1991; Paget et al., 1992; Khanna and Stotzky, 1992; Paget and Simonet, 1994; Gallori et al., 1994; Poly et al., 2000). Other abiotic factors influencing the adsorption of nucleic acids to soil particles are pH and the availability of bivalent ions. Studies on the persistence of free DNA in soil have often been performed in rather artificial soil model systems, e.g. sand or sterile soil. Only recently reports on the persistence of DNA in nonsterile soil have been published (Widmer et al., 1996; Nielsen et al., 1997; Blum et al., 1997; Gebhard and Smalla, 1999). Microbial activity was pinpointed as an important biotic factor affecting the persistence of free DNA in soil (Blum et al., 1997). Stimulated microbial activity often coincided with an increase in DNase activity in soil (Blum et al., 1997).

Recently it was shown that DNA is also well protected in dead cells in soil and that this DNA still has transforming activity (Nielsen et al., 2000a). Nielsen et al. (2000a) could show that cell lysates of *Pseudomonas fluorescens*, *Burkholderia cepacia* and *Acinetobacter* spp. were available as a source of transforming DNA for *Acinetobacter* sp. populations in sterile and nonsterile soil for a few days and that cell debris protect DNA from inactivation in soil. Cell walls might play an important role in protecting DNA after cell death (Paget and Simonet, 1997). Contact between dead or intact donor and competent recipient cell membranes might facilitate binding and take-up of the DNA (Paul et al., 1992; Paget and Simonet, 1997).

Field releases of transgenic rizomania-resistant sugar beet plants were accompanied by studies on the persistence of DNA from sugar beet litter in soil. To detect transgenic DNA independently from cultivation, total community DNA was extracted directly from soil and amplified with three different primer sets specific for transgenic DNA. Parts of the construct were detectable for up to 2 years, and also in soil microcosms with introduced free DNA long-term persistence could be shown (Gebhard and Smalla, 1999). Under field conditions, it may well be that transgenic plant DNA is protected by still intact plant cells for quite some time. When we studied the persistence of free plant DNA introduced into soil under microcosm conditions, we observed a rapid initial decrease of the levels of transgenic DNA as evidenced by direct hybridization of the community DNA with probes specific for the construct. However, by PCR the transgenic construct was detectable for several months. Long-term persistence of transgenic plant DNA was also observed by Widmer et al. (1996, 1997) and by Paget and Simonet (1997) under microcosm and field conditions. A more rapid decrease of transgenic DNA was observed at higher soil humidity and temperature. Both factors are supposed to contribute to a higher microbial activity in soil (Widmer et al., 1996; Blum et al., 1997). Since plant DNA can persist adsorbed on soil particles or protected in plant cells, this DNA could be captured by competent bacteria colonizing in close vicinity.

Can soil and plant-associated bacteria capture antibiotic resistance genes from transgenic plants via a transformation process?

Long-term persistence, even of a small percentage of the released plant DNA, is assumed to enhance the likelihood of transformation processes. Furthermore, it was hypothesized that the introduction of bacterial genes, promotor and terminator sequences into the plant genome might lead to an enhanced probability of gene transfer events from transgenic plants to bacteria. However, until recently it was completely unclear whether bacteria could be transformed by plant DNA at all. Several groups had failed to detect horizontal gene transfer from transgenic plants to bacteria, perhaps because of an absence of homologous sequences in the bacteria (Nielsen et al., 1997c) or the use of less efficiently transformable bacteria (Schlüter et al., 1995; Broer et al., 1996). Another reason for previous failures to detect horizontal gene transfer from plants to bacteria was that the transfer of complete genes was monitored, whereas bacterial transformation might involve recombination of short DNA segments. Furthermore, the high content of non-bacterial DNA and the much higher methylation rate were

supposed to prevent the transfer of antibiotic resistance genes from transgenic plant DNA to bacteria.The ability of *Acinetobacter* sp. BD413 pFG4Δ*npt*II to capture and integrate transgenic plant DNA based on homologous recombination could be demonstrated under optimized laboratory conditions (Gebhard and Smalla, 1998). Restoration of a 317 bp deletion resulting in kanamycin resistant *Acinetobacter* sp. BD413 was observed not only with transgenic plant DNA but also with transgenic plant homogenates (Gebhard and Smalla, 1998). De Vries and Wackernagel (1998) reported that transformation of competent *Acinetobacter* sp. BD413 cells containing an *npt*II-gene with a 10 bp deletion, with DNA from various transgenic plants (*Solanum tuberosum*, *Nicotiana tabacum*, *Beta vulgaris*, *Brassica napus*, *Lycopersicon esculentum*) carrying a *npt*II as marker gene, resulted in the restoration of the deleted *npt*II gene. Both studies had in common that *Acinetobacter* sp. BD413 was transformed and that *npt*II gene carrying deletions served for sequence homology and as a detection system (restoration of the *npt*II gene resulted in a kanamycin resistance phenotype). However, when transgenic potato plants carrying an *npt*II gene were colonized by *Ralstonia solanacearum* pFG4 Δ*npt*II, the causative agent of potato wilt disease, no restoration of the *npt*II gene could be detected (Borin et al., unpublished). This preliminary result might indicate that *Ralstonia solanacearum* is much less efficiently transformable than *Acinetobacter* sp. BD413 (Bertolla et al., 1999; Borin, unpublished). Another strain of *Ralstonia solanacearum* was reported to develop competence *in planta* and to exchange genetic information *in planta* (Bertolla et al., 1997, 1999). However, gene exchange was demonstrated when tomato plants infected with *Ralstonia solanacearum* were inoculated with plasmid DNA or during co-infection with *R. solanacearum* carrying different genetic markers, and not during colonization of transgenic plants. A new finding concerning horizontal transfer of antibiotic resistance genes from plants to bacteria is that we have experimental proof now that DNA from transgenic plants can be captured by bacteria based on homologous recombination. But how to interpret this scientifically interesting finding? First of all, it is important to look at the frequencies of these transfer events. Compared to transformation with chromosomal or plasmid DNA, transformation frequencies with plant DNA or plant homogenates were drastically reduced when the experiments done by filter transformation of *Acinetobacter* sp. BD413 pFG4 were taken a step further and performed in sterile and nonsterile soil. Transformation of *Acinetobacter* sp. BD413 pFG4 by transgenic sugar beet DNA could be detected in sterile but not in nonsterile soil (Nielsen et al., 2000b). The authors estimated that frequencies of transformants in nonsterile soil would be at 10^{-10} to 10^{-11} and thus below the level of detection. Experimental studies have confirmed the low probability of integration of transgenes in the bacterial genome of the recipient if DNA homology is not present (Nielsen et al., 1997c). Studies on gene transfer by natural transformation have revealed that additive integration of nonhomologous genetic material can occur when flanking homology is present (Nielsen et al., 1997a,b). Present data suggest that transformation of competent bacteria by transgenic plant DNA in soil and in the rhizosphere occurs if at all at very low frequencies (Gebhard and Smalla, 1999; Nielsen et al., 2000b). However, it cannot be ruled out that hot spots, such as the digestive tract of insects, exist which might promote gene transfer events (Hoffmann et al., 1998; 1999).

Among the different steps in the process of natural transformation, the release of DNA from cells, its persistence and its availability to be taken up by competent bacteria have been well documented (Paget and Simonet, 1997). The major limiting factor for natural transformation remains the presence of competent bacteria and the development of competence. In most studies on transformation, competent bacteria have been inoculated in the soil system studied (Gallori et al., 1994; Nielsen et al., 1997a; Sikorski et al., 1998). Only recently, Nielsen et al. (1997b; 2000b) could show that noncompetent *Acinetobacter* sp. strain BD413 cells residing in soil could become competent after addition of nutrients. Nutrient solutions used to stimulate competence development in *Acinetobacter* sp. BD413 populations contained inorganic salts and simple compounds corresponding to rhizosphere exudates (Nielsen et al., 2000b). Presently, we still know very little about the importance of transformation processes in environmental habitats. In contrast, HGT by conjugation or mobilization under different environmental conditions is much better documented (Thomas and Smalla, 2000).

It cannot be excluded that HGT from plants to bacteria may take place in different environmental niches but the ecological significance of such rare events depends upon the selection of the acquired trait and the current dissemination of respective antibiotic resistance genes. For more comprehensive reading on natural transformation, several recently published reviews are recommended (Lorenz and Wackernagel, 1994; Nielsen et al., 1998; Bertolla and Simonet, 1999).

Is prevalence and dissemination of antibiotic resistance correlated with antibiotic selective pressure?

Bacterial antibiotic resistance is increasingly limiting the therapeutic use of antibiotics to treat and control many types of bacterial diseases. The extent of antibiotic use over the last 50 years has altered

the composition of microbial communities. Bacterial populations resistant to the antibiotics applied obviously gained a selective advantage in comparison to the sensitive populations. Shifts in microbial communities as a consequence of antibiotic selective pressure are often linked with intrinsic or acquired resistance to the antibiotic used. Several studies clearly demonstrated a correlation between the application of antibiotics and the incidence of the respective antibiotic resistance genes (Levy 1987, 1997; Tschäpe, 1994; Witte, 1997, 1998). In contrast to the use of antibiotics by man, many rhizobacteria such as *Streptomycetes* spp., *Erwinia carotovora* and *Pseudomonas aureofaciens* produce antibiotics in a very sophisticated manner, i.e. via quorum sensing (McGowan et al., 1995; Pierson et al., 1998). Only when high population densities are reached, is the expression of antibiotic producing genes activated. Antibiotic producing strains also require the respective antibiotic resistance genes and thus might be a source of antibiotic resistance genes acquired by human pathogens.
The emergence of bacterial antibiotic resistances as a consequence of the wide-scale use of antibiotics by humans has resulted in a rapid evolution of bacterial genomes. Mobile genetic elements such as transferable plasmids, transposons and integrons have played a key role in the dissemination of antibiotic resistance genes amongst bacterial populations and have contributed to the acquisition and assembly of multiple antibiotic resistance in bacterial pathogens (Tschäpe, 1994; Salyers and Shoemaker, 1994; Witte, 1998). Since bacteria circulate between different environments and different geographic areas, the global nature of the problem of bacterial antibiotic resistances requires that data on their prevalence, selection and spread are obtained in a more comprehensive way than before.
The incidence of bacteria resistant to one or to multiple antibiotics has been traditionally studied by plating on antibiotic containing nutrient media or by screening bacterial isolates for their antibiotic resistance patterns. However, various different mechanisms can be responsible for a decreased sensitivity towards antibiotics (Shaw et al., 1993). Molecular tools which have become increasingly available in the eighties allowed great progress in the study of the epidemiology of bacterial antibiotic resistance at the genetic level. DNA hybridization and sequencing revealed that different genes can code for the same function. The important contribution of mobile genetic elements, such as plasmids, conjugative transposons, integrons and gene cassettes, to bacterial genome plasticity and adaptation was demonstrated by several groups (Tschäpe, 1994; Salyers and Shoemaker, 1994; Hall, 1997; Thomas and Smalla, 2000). DNA probes and PCR-based detection systems allow us not only to analyze the dissemination of antibiotic resistance genes in the culturable fraction of bacteria but also to extend our knowledge to the majority of bacteria which are not accessible to traditional cultivation techniques (Smalla and van Elsas, 1995). Only a few studies have provided data on the prevalence of antibiotic resistance genes used as markers in transgenic plants. Studies on the dissemination of the most widely used marker gene, *npt*II, in bacteria from sewage, manure, river water and soils demonstrated that in a high proportion of kanamycin-resistant enteric bacteria the resistance is encoded by the *npt*II-gene (Leff et al., 1993; Smalla et al., 1993). In the framework of an EU-BIOTECH project RESERVOIR (http:\\www.reservoir.dk) a comprehensive multiphasic survey of the prevalence and transfer of antibiotic resistance genes (e.g. genes conferring resistance to tetracycline or streptomycin) in different environments is presently being performed. This study includes samples from soils, rhizospheres, manure from cattle, swine and chicken, municipal sewage, and coastal water. From these environments, streptomycin and tetracycline resistances located on transferable elements could be isolated into different bacterial hosts using exogenous plasmid isolation techniques (unpublished results of the RESERVOIR group).
Bacteria resistant to multiple antibiotics are not restricted to clinical environments but can easily be isolated from different environmental samples and food (Perreten et al., 1997; Feuerpfeil, 1999; Dröge et al., 2000). The traditional perception that in some way the hospital environment is closed and that antimicrobial resistance arises in patients in hospitals needs to be corrected. There is substantial movement of antibiotic resistance genes and antibiotic resistant bacteria between different environments. In assessing the antibiotic resistance problem, a number of factors can be identified which have contributed to the antibiotic resistance problem: the antibiotic itself and the antibiotic resistance trait (Levy, 1997). The genetic plasticity of bacteria has largely contributed to the efficiency by which antibiotic resistance has emerged. However, horizontal gene transfer events have no *a priori* consequence unless there is antibiotic selective pressure (Levy, 1997).

Conclusion

Given the fact that antibiotic resistance genes, often located on mobile genetic elements, are already widespread in bacterial populations and that HGT events from transgenic plants to bacteria are supposed to occur at extremely low frequencies and have not yet been detected under field conditions, it is unlikely that antibiotic resistance genes used as markers in transgenic crops will contribute significantly to the spread of antibiotic resistance in bacterial populations. There is no doubt that the present problems in human and veterinary medicine, resulting from the selective pressure

posed on microbial communities, were created by the unrestricted use of antibiotics in medicine and animal husbandries, and not by transgenic crops carrying antibiotic resistance markers. Unfortunately, in some European countries the discussion about antibiotic resistance genes in transgenic crops attracts much more public attention than the massive use of antibiotics. We feel that the public debate about antibiotic resistance genes in transgenic plants should not divert the attention from the real causes of bacterial resistance to antibiotics such as the continued abuse and overuse of antibiotics by physicians and veterinarians (Salyers, 1996). The control of the antibiotic resistance problem very clearly lies in a reduction of the selective pressure by prudent use of antibiotics.

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