Opinion of the Scientific Panel on Genetically Modified Organisms on the use of antibiotic resistance genes as marker genes in genetically modified plants[1] (Question N° EFSA-Q-2003-109)

Opinion adopted on 2 April 2004

SUMMARY

Directive 2001/18/EC (EC, 2001) states that Member States and the Commission shall ensure that GMOs which contain genes expressing resistance to antibiotics in use for medical or veterinary treatment are taken into particular consideration when carrying out an environmental risk assessment. This is with a view to identify and phase out antibiotic resistance marker genes (ARMGs) in GMOs which may have adverse effects on human health and the environment.

The Scientific Panel on genetically modified organisms (GMO Panel) of the European Food Safety Authority (EFSA) has evaluated the potential risks associated with specific ARMGs taking into account their current usage in clinical and veterinary medicine, the likely occurrence of horizontal gene transfer from genetically modified (GM) plants to microbes and the potential impact of horizontal gene transfer where naturally occurring resistance to the relevant antibiotics exists in the microbial gene pool. These factors will impact on the likelihood of any adverse effects on humans or the environment of ARMGs used in GM plants.

The GMO Panel considers the frequency of horizontal gene transfer from GM plants to other organisms as very low for all ARMGs considered. This, in itself, is an important consideration with regard to any risk posed by the use of ARMGs. However, with respect to clinical importance the Panel has categorised ARMGs into three groups with different potentials for compromising human health and the environment. ARMGs in the first group include genes conferring resistance to kanamycin and hygromycin. In this group the *npt*II gene, which confers kanamycin resistance, has a 13-year history of safe use in food crops and resistance to this group of antibiotics is widespread in naturally occurring microbes in humans and the environment. The Panel is of the opinion that with regard to safety there is no rationale for inhibiting or restricting the use of genes in this category, either for field experimentation or for the purpose of placing on the market. The second group of ARMGs, which includes resistance to chloramphenicol, ampicillin, streptomycin and spectinomycin, should be restricted to field trial purposes and should not be present in GM plants to be placed on the market. Given their current importance in clinical usage, the GMO Panel recommends that ARMGs placed in the third group, which includes those conferring resistance to amikacin and tetracyclines, are not present in GM plants to be placed on the market or in plants used for experimental field trials.

Keywords: Directive 2001/18/EC, GMOs, GM plants, antibiotics, antibiotic resistance marker genes, safety, human health, environment, horizontal gene transfer, *npt*II gene.

[1] For citation purposes: Opinion of the Scientific Panel on Genetically Modified Organisms on the use of antibiotic resistance genes as marker genes in genetically modified plants, *The EFSA Journal* (2004) 48, 1-18

TABLE OF CONTENTS

BACKGROUND

Directive 2001/18/EC of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms (EC, 2001) lays down in Annex III the information which may be necessary to carry out the environmental risk assessment. Article 4 (2) of the Directive states that Member States and the Commission shall ensure that GMOs which contain genes expressing resistance to antibiotics in use for medical or veterinary treatment are taken into particular consideration when carrying out an environmental risk assessment. This is with a view to identify and phase out antibiotic resistance marker genes (ARMGs) in GMOs which may have adverse effects on human health and the environment. This phasing out shall take place by the 31 December 2004 in the case of GMOs placed on the market according to Part C of the Directive and by 31 December 2008 in the case of GMOs authorised for experimental releases under Part B of the Directive. Annex II of Directive 2001/18/EC states that the risk assessment of the use of antibiotic resistance marker genes is a very specific issue and that further guidance may be recommended.

In accordance with Directive 2001/18/EC, a notification for placing on the market a GMO that has received a positive assessment report from a lead Member State is transmitted to the competent authorities of other Member States which can raise objections to the proposed marketing of the GMO during the statutory 60-day period and discuss outstanding issues for a further 45 days. Where objections are maintained, the Commission is required to consult the relevant Scientific Committees, now represented by the European Food Safety Authority (EFSA). The presence of ARMGs in the notified GMO products is often a reason for such objections.

The GMO Panel has recognised the need for guidance to notifiers, the Member States, and the Commission for identifying antibiotic resistance genes with the potential to be used as marker genes for GM plants and which may or may not have adverse effects on human health and the environment. The GMO Panel is aware of the limited availability of alternative marker genes for GM plants and of the ongoing development of marker removal systems. Future activities of the GMO Panel might focus on these alternatives in the event that more data become available.

TERMS OF REFERENCE

Recognising the importance and urgency of the question, the GMO Panel has decided to task itself to deliver a scientific opinion on:

antibiotic resistance genes with the potential to be used as marker genes for genetically modified plants and which may or may not have adverse effects on human health and the environment taking into account the limited availability of alternatives.

The GMO Panel set up a Working Group on ARMGs to provide a scientific opinion in due time for the ongoing activity of the Working Group of Committee of the Competent Authorities under Directive 2001/18/EC on the implementation of Article 4 (2) of Directive 2001/18/EC.

ASSESSMENT

1. Introduction

During the process of genetic modification of plants and other organisms, marker genes are normally used to facilitate the selection and identification of genetically modified cells, containing the gene of interest inserted into the genome of the host organism, among the vast majority of untransformed cells. This opinion deals solely with the use of antibiotic resistance marker genes (ARMGs) as particular concerns have been raised over the use of such genes and the potential for increased resistance to antibiotics in humans and animals as a result of horizontal gene transfer. The use of ARMGs has been common practice in microbial genetic research for many years and their utility has been extended successfully to the genetic modification of plants, including agricultural crops. ARMGs which may differ from those used to select the final transgenic plants are used in the initial molecular cloning procedures for construct development, an approach usually performed in micro-organisms. Since these genes can also be incorporated into the target plant the risk associated with the presence of these ARMGs needs to be considered alongside the ARMGs used specifically for the selection of successfully transformed cells.

GM plants approved for placing on the market and intended for unrestricted use by third parties might become widely distributed and used. A concern with respect to ARMGs is the theoretical possibility that the clinical therapy of orally administered antibiotics could be compromised through inactivation by antibiotic resistance proteins present in food derived from a GM plant containing an ARMG. The efficacy of antibiotic therapy is related to the topic of human and animal safety. Safety of ARMGs should be considered taking into account the following aspects: 1) prevalence of resistance to the antibiotic among bacteria in the intestine or in the environment and 2) the extent of use of the antibiotic and its importance for clinical human/animal therapy. Although there is no evidence that the presence of ARMGs in GM plants has caused any damage, it might be advisable to restrict the use of specific ARMGs. In this document, the key issues related to the biosafety of the use of ARMGs in GM plants are considered (reviewed by Bennett *et al*., 2004).

2. Prevalence of antibiotic resistance genes in nature

Antibiotic resistance is a relatively common feature in natural microbial communities for a range of different habitats such as soils, aquatic systems and animal- and human-associated habitats. In fact, the majority of antibiotics currently used are produced in nature by micro-organisms (e.g. streptomycins are produced by streptomycetes), and the micro-organisms producing antibiotic themselves also contain the corresponding antibiotic resistance genes for

self-protection. The production of antibiotics is thought to represent a defence mechanism against competing micro-organisms, and is thus a key survival mechanism in nature. The mechanism(s) conferring antibiotic resistance in micro-organisms can vary, including options such as (1) enzymatic inactivation or modification of the antibiotic, (2) modification of host targets to prevent antibiotic binding, (3) failure of the antibiotic to be transported into and/or to be maintained in the micro-organism.

In addition to the presence of antibiotic resistance genes in the antibiotic-producer organisms, these genes also occur in natural bacterial assemblages in the so-called "horizontal gene pool", i.e. the fraction of genes in the bacterial population that is carried on mobile genetic elements such as plasmids and conjugative transposons. The horizontal gene pool provides flexibility to natural bacterial communities by protecting against the effect of antibiotics at times when antibiotic selective pressure is common in the local habitat.

During the last fifty years, antibiotic-resistant micro-organisms, in particular bacteria, have become prevalent in hospital- and/or patient-associated environments as a result of the ever-increasing use of antibiotics in clinical environments. Horizontal gene transfer and clonal selection are key genetic processes in these microbial populations. This scenario is true for each new antibiotic introduced. In addition, high prevalence of antibiotic resistance has been observed (in particular, tetracycline resistance) due to the use of antibiotics in agriculture, e.g. as feed additives. Resistance can persist in animal populations for a long period of time when the antibiotic is no longer used (Hinton *et al.*, 1986). A European survey of a range of habitats (including soils, waste water, and plant-associated habitats), performed under the EU research project RESERVOIR[2], showed that streptomycin, gentamycin and tetracycline resistances were widely spread in all environments tested, irrespective of whether the antibiotics were released into these environments (Heuer *et al.*, 2002; Van Overbeek *et al.*, 2002).

3. Antibiotic use and its effect on the antibiotic resistance gene pool

Pools of antibiotic resistance genes are thought to have been naturally present in microbial communities in natural and man-associated environments for a considerable period of time. The prevalence of these genes in natural microbial communities is strongly associated with the balance between the gain of fitness under selective pressure and the fitness loss under non-selective conditions. The outcome of this balance is unsure, often unpredictable and depends on the mechanisms involved. Rapid loss of antibiotic resistance from natural microbial communities has been observed in certain cases, e.g. for streptomycin resistance, but there are also examples of the persistence of the resistance trait, e.g. tetracycline resistance (Hinton *et al.*, 1986).

The increasing incidence of antibiotic resistance in microbial assemblages associated with environments important to humans and animals, in particular when present in the horizontal gene pool, poses an obvious risk to the ability of man to control pathogens in the clinical environment. This situation is difficult to solve, especially in the light of the potential persistance of antibiotic resistance in natural bacterial communities and the different mechanisms that can give rise to antibiotic resistance. In any case, prudent use of antibiotics will be the major strategy (Salyers, 1996).

4. The potential impact of horizontal gene transfer

The transfer of DNA, e.g. antibiotic resistance marker gene, from GM plant material to bacteria and the potential consequences are relevant issues to consider. Gene flow amongst bacteria is a

[2] BIO4-CT98-053, Antibiotic resistance genes in the environment: a comprehensive, multi-phasic survey of prevalence and transfer

well-established natural process that is recognised as central to their survival and evolution. This is especially well illustrated in the development of multiple drug resistance, a phenomenon that has been analysed in great detail and demonstrates the prominence of gene transfer and DNA rearrangement in bacteria. In contrast, there is no *a priori* reason to expect gene flow from plants to bacteria. Generally, it is accepted that the mechanism for such a transfer event would be the capture of DNA released from GM plant material by competent bacteria via natural transformation.

Thus, an important factor may be the persistence of plant-derived DNA in the environment during crop cultivation and harvesting (and in soil residues), during food processing and in the human or animal gastro-intestinal tract. GM plant material intended for use in food is often subject to a variety of processing regimes. These range from simple heat treatment (e.g. canning) to the extraction of food ingredients. Food processing and extraction of ingredients may physically damage, degrade or remove DNA and this limits gene transfer. Published studies on the susceptibility of DNA to processing and extraction regimes have been reviewed (Klein *et al.*. 1998). The gastro-intestinal tracts of man and animals degrade DNA and destroy intact biologically active genes (Beever and Kemp 2000). However the process of inactivation is not complete, especially in the more proximal regions of the gastro-intestinal tract.

Experimental studies on the fate of DNA involve detection using PCR amplification and the assessment of biological activity/integrity of DNA by transformation studies. Mercer *et al*. (1999; 2001) investigated DNA degradation in the human oral cavity and demonstrated that although DNA was rapidly degraded, sufficient biological activity remained to allow transformation of competent *Streptococcus gordonii* cells. Duggan *et al*. (2000) investigated sheep saliva and rumen fluid, concluding that DNA remained available for transformation in the oral cavity but was rapidly inactivated further down the gastro-intestinal tract. Duggan *et al*. (2003) investigated maize grains and found that the cellular matrix protected DNA from degradation. Martin-Orue *et al.* (2002) found that DNA in food was degraded much slower than pure DNA. Chambers *et al*. (2002) used chicken feeding experiments to explore the *in vivo* fate of the bacterial ampicillin resistance gene *bla* in bacteria and transgenic maize. The gene was found in the stomach contents when GM maize was fed to chickens but not in the lower intestine. In contrast, feeding bacteria containing *bla* led to the detection of the gene throughout the intestinal tract. Netherwood *et al.* (2002) used human ileostomists to monitor the survival of transgenes in GM plant material during passage through the human gastro-intestinal tract. Transgene survival was detected in the small intestine but in a trial using human volunteers with an intact gastro-intestinal tract no transgenic DNA was detected in their faeces.

Various experiments (Schubbert *et al*., 1994; 1997; 1998; Hohlweg and Doerfler, 2001; Klotz and Einspanier, 1998; Einspanier *et al*., 2001) have demonstrated that pure DNA as well as plant-associated DNA, when consumed in a diet, can sometimes be detected in very low amounts in blood and tissues. There is no reason to expect differences in the fate of DNA derived from GM plants and non-GM plants. It is very well established that some bacterial species possess highly evolved processes that allow them to take up DNA from the environment (Lorenz and Wackernagel, 1994). However, the development of this 'competence' is a regulated process that depends on particular environmental circumstances. Bacteria produce restriction endonucleases that degrade incoming foreign DNA and, to be maintained, DNA that is not degraded must be capable of replication. This depends either on the presence of a genetically linked replicon or an integration event. In characterised natural transformation systems (Chen and Dubnau, 2003) DNA is taken into the cell as a single strand. Efficient integration depends on DNA homology between incoming DNA and the recipient bacterial genome (Lewin, 2000) or on a site-directed integration mechanism, the latter being highly specific. Integration can also involve a rare 'illegitimate' recombination event. Thus, the provision of DNA homology between the transgene and the recipient bacterial genome will facilitate plant to bacterium DNA transfer (Gebhart and Smalla, 1998; Nielsen *et al*., 1998, 2000). The DNA acquired by the bacterium is unlikely to be of significance unless it is expressed or alters the expression of resident genes.

Bacterial gene expression depends on specific genetic signals that are not universal between species providing another molecular barrier.

DNA transfer from GM plant material to micro-organisms has been investigated in a limited number of experimental studies. Schluter *et al*. (1995) used *Erwinia chrysanthemi* as a recipient in experiments with transgenic potato. The latter carried a complete copy of a bacterial plasmid capable of replication and marker gene expression in *Erwinia*. This pathogen lyses plant tissues with extracellular pectinolytic enzymes and thus has an intimate association with the plant material. Despite this, evidence for plant to bacterium transfer was not found. Gebhard and Smalla (1998) and de Vries and Wackernagel (1998) used naturally competent *Acinetobacter* to investigate plant to bacterium gene transfer by marker rescue. This process depends on the presence of DNA homology between the transgene and recipient bacterium and transformation involves the correction of a mutation by homologous recombination. In all cases, the plant material carried an *npt*II kanamycin resistance gene and the recipient bacteria carried an inactivated homologue of the same gene but controlled by a bacterial promoter. Transformants could only be detected when the *npt*II gene in the bacteria was restored. When the DNA homology between donor and recipient was removed, transformation fell below the limit of detection, suggesting the absence of adventitious degrees of homology between the integration and the recipient genome (Nielsen *et al*., 1998). Thus, whilst there is evidence for gene transfer by marker rescue, the recovery of unique DNA from the transgenic plant was not demonstrated. There is a similar report of marker rescue using GM potatoes with *Acinetobacter* and *Pseudomonas stutzeri* (De Vries *et al*., 2001). Nielsen *et al*.. (2000) extended the findings on marker rescue to soil and Kay *et al*. (2002) included studies in GM plants in which transgenic DNA was integrated within the chloroplast genome.

Thus, horizontal gene transfer from plants to micro-organisms is possible, but with a low frequency when enforced under specific experimental conditions conducive to the gene transfer process (e.g. the presence of homology between sequences flanking the transgenic DNA and the genome of the recipient bacterium). The frequency is apparently very low under natural circumstances (Nielsen *et al*., 2000; Kay *et al*., 2001). A current EU sponsored project TRANSBAC[3] attempts to map the occurrence of anchor sequences across plant and bacterial genomes.

5. Antibiotic resistance genes with marker function in plants

5.1. Kanamycin resistance: *npt*II gene

The *npt*II [= aph(3´)-IIa] gene is widely used as a selectable marker (often referred to as kanamycin resistance gene or neomycin resistance gene) in the transformation of organisms as diverse as bacteria, yeasts, plants and animals. It was the first marker used in plant genetic transformation and is still the most commonly used marker in the selection of transformed plants. Kanamycin is normally used as the selective agent for the *npt*II gene.

Origin - The gene originates from the transposon Tn5 of *Escherichia coli* K12 (Garfinkel *et al*., 1981).

Catalytic activity and substrate specificity - The gene encodes neomycin phosphotransferase. Neomycin phosphotransferase is a type II aminoglycoside-3'-phosphotransferase (APH (3')II) catalyzing an ATP-dependent phosphorylation of the 3'-hydroxyl group of the aminohexose moiety of certain aminoglycoside antibiotics (Bryan, 1984). The modified kanamycin molecule can no longer bind to the 30S ribosomal subunit to cause misreading of mRNA and thus inhibit

[3] QLK3-2001-02242, Gene flow from transgenic plants: evaluation and biotechnology.

protein synthesis. Since the phosphorylation is ATP-dependent, ATP has to be present in sufficient amounts for the catalytic reaction to take place. The APH(3')II protein has the antibiotics kanamycin, neomycin, paromomycin, ribostamycin, butirosin, gentamicin B and geneticin (G418) as substrates and renders the carrier of the trait resistant to these antibiotics. Resistance has not been conferred for amikacin, but enzymatic activity for this substrate is detectable *in vitro*. The marker gene commonly used in genetic modifications of plants encodes an aminoglycoside 3'-phosphotransferase that confers resistance only to the antibiotics neomycin, kanamycin and geneticin (as reviewed by Redenbaugh *et al*., 1993, 1994). Mutations in the *npt*II gene may result in modifications of the amino acid sequence of the protein that may eliminate, reduce or increase aminoglycoside resistance or lead to an alteration in the substrate specificity of the enzyme. For instance, a point mutation in the *npt*II gene modified the specificity of the enzyme conferring the ability to phosphorylate amikacin (Kocabiyik and Perlin 1992). However, no amikacin resistant strains with clinical significance have been obtained so far by introducing a single mutation in the *npt*II gene. Resistance which is clinically significant could only be obtained under laboratory conditions and required two simultaneous and rare mutation events affecting two different genes, *npt*II and a gene encoding a permease (Perlin and Lerner, 1996). The natural occurrence of such double mutants has not been reported, while resistance to gentamycin, amikacin and tobramycin caused by the presence of a number of other antibiotic resistance genes is widespread (Schmitz *et al*., 1999).

Therapeutic importance of the relevant antibiotics *(kanamycin, neomycin, geneticin)* – Kanamycin is rarely used today because of its considerable side effects. Only under conditions of multiple mycobacterial resistance to other drugs is kanamycin still used as a reserve tuberculostatic agent. For the same reason as kanamycin, neomycin, which is poorly absorbed orally, is also rarely used intravenously/intramuscularly to treat infections. Neomycin is sometimes used orally for pre-operative bowel sterilization, for selective gut decontamination in certain high-risk patients, or for the treatment of hepatic encephalopathy. Kanamycin and neomycin are also components in some formulations used for localised treatments of infections in skin, eyes and ears. These antibiotics are rarely administered orally, which minimizes the selective pressure for antibiotic resistance in the gut. Their use in the treatment of humans has been superceeded by more effective aminoglycoside antibiotics that are not substrates for APH(3')-II (Nap *et al.,* 1992). However, neomycin has some veterinary use, primarily to treat calves and pigs (and poultry) for intestinal infections (enteritis). It is also used for the treatment of bacterial skin infections, including dermatitis and eczema in cats and dogs. The antibiotics are rarely used in agriculture or aquaculture and thereby do not provide selective pressure for a possible transfer of the resistance genes from genetically modified plants to soil micro-organisms. In contrast to kanamycin and neomycin, geneticin is only used for *in vitro* experimentation e.g. as a selective agent for eukaryotic GM cells.

Resistance occurrence – Kanamycin as well as neomycin resistant bacteria are ubiquitous in nature. Selective plating of soil bacteria on kanamycin-containing medium can reduce the microbial count from 10^7 to 10^4 CFU/g (Smalla *et al*., 1993; Smalla and van Elsas 1996). However, only a fraction of kanamycin resistant bacteria often contain the *aph*(3')-IIa gene. The other resistant bacteria have other genes conferring kanamycin resistance. At least seven isozymes of APH (3´) have been reported in the literature. The *aph*(3')-IIa gene which encodes the APH (3´)-IIa protein has been reported to occur naturally only in eubacteria. The gene occurs in gram-negative organisms and *Pseudomonas* spp. In one survey, three out of 350 kanamycin resistant bacterial isolates from different soils, river water, sewage and pig slurry contained *aph*(3')-IIa sequences (Smalla *et al*., 1993). The organisms belonged to the Proteobacteria, being classed as *Aeromonas* spp. and *Escherichia coli*. Leff *et al*. (1993) showed similar data (3/184 positives) for stream isolates. In a survey of over 4200 clinical isolates resistant to one or more aminoglycoside antibiotics, 2.5% of the bacteria contained the *aph*(3')-IIa sequences. The data emphasise that, although there is a great diversity of genes encoding aminoglycoside-modifying enzymes, most of these genes are currently restricted to gram-negative bacteria. This phenomenon may be due to different requirements for gene expression, plasmid replication,

and barriers of genetic exchange. Aminoglycoside resistant bacterial strains often emerge as a result of acquiring plasmid-borne genes encoding aminoglycoside-modifying enzymes (Courvalin and Carlier 1981). Furthermore, many of these genes are associated with transposons, which aid the rapid dissemination of drug resistance. Using worst case probability estimates for hypothetical gene transfer, it has been concluded that the additive effect of an *aph*(3')-IIa gene-containing DNA fragment entering the human gastrointestinal flora from genetically engineered plants is insignificant in terms of gaining a selective advantage when compared to the population of kanamycin resistant micro-organisms naturally present.

Other safety considerations - The purified enzyme has been shown to be rapidly degraded in studies simulating normal gastric and intestinal conditions (Fuchs *et al*., 1993a, b); the protein degraded in 10 seconds and no enzymatic activity was found after 5 min. Thus, in the stomach and small intestine, most, if not all, APH (3')II protein will be inactivated or degraded by the acidic environment and digestive enzymes. Under simulated abnormal conditions in neutralized gastric fluid (which may exist in patients treated with drugs that reduce stomach acidity) the enzyme may remain active. Even if not degraded, APH (3')II would not function under the limited concentration of ATP present. Using GM tomato expressing the APH (3')II marker protein as an example, and assuming that the tomato was eaten together with 1 g of relevant antibiotic (neomycin), loss of antibiotic efficacy would be maximally only 1.5% (Redenbaugh *et al*., 1993, 1994). The number is based on the following assumptions: 1) 95th percentile consumption[4], at a single serving, of specific fruits or vegetables high in ATP content; 2) calculations based on a survey of a three-day consumption period; 3) stoichiometric reaction of 100% of the ATP in ingested food with orally administered neomycin; 4) administration of neomycin simultaneously with consumption of a GM food containing APH(3')-II and other fruits or vegetables rich in ATP; 5) presence of intact, functional APH(3')-II enzyme, which requires a buffered stomach environment (pH 7); and 6) stability of ATP in the stomach environment. The conclusion was that there is no risk of compromising efficacy of oral therapeutic use of kanamycin and neomycin due to APH(3')-II present in food (Redenbaugh *et al.,* 1993, 1994).

5.2. Hygromycin resistance: *hph* gene

Origin - The *hph* [= *aph*(4)Ia] gene originates from *Escherichia coli* W677 carrying the plasmid pJR225. Two major genes encoding Hph protein have been characterized. The first gene was isolated from *Streptomyces hygroscopicus* (Leboul and Davies, 1982; Malpartida *et al*., 1983), a hygromycin B producing species. The second gene is a plasmid-borne resistance gene isolated from *Escherichia coli* (Rao *et al*., 1983; Kuhstoss and Rao, 1983) and *Klebsiella pneumoniae* (Gritz and Davis, 1983). Most vectors containing resistance genes and used in gene transfer experiments with plants harbour the *E. coli hph* gene.

Catalytic activity and substrate specificity - The *hph* gene encodes hygromycin phosphotransferase (Waldron *et al*., 1985). The protein of this gene inactivates, specifically, the antibiotic hygromycin B by phosphorylation (Gritz and Davies, 1983). Other aminoglycoside aminocyclitol antibiotics such as kanamycin or geneticin are not substrates for the enzyme. The *hph* gene is 1023 bp and rich in CpG dinucleotides (103 CpG). To avoid any *hph* gene silencing in eukaryotic expression vectors due to the high proportion of CpG dinucleotides, a functional synthetic *hph* gene is available. In the synthetic gene all of the CpG motifs have been removed and the codon usage optimised. The synthetic *hph*-DCpG gene displays higher hygromycin resistance than its wild-type counterpart.

Therapeutic importance – Hygromycin is not in human clinical use but may be used in veterinary medicine for treatment of swine and poultry (USA, not licensed in the UK).

[4] Confidence interval, in which the true mean value can be found with a likelyhood of 95%

Resistance occurrence – No systematically published information available.

5.3. Streptomycin resistance: *aad*A (Strep/Spec^R) gene

Origin – The *aad*A [= *ant*(3'')-Ia, Strep/Spec^R] gene originates from the plasmid R538-1 of *Escherichia coli.* The gene is ubiquitous among gram-negative bacteria and has been cloned from several transposons. Tomalsky and Crosa (1987) detected the aadA (Strep/Spec^R) gene on the multiresistance transposon Tn1331 in *Klebsiella pneumonia*.

Catalytic activity and substrate specificity – The gene encodes streptomycin adenyltransferase (Davies and Smith, 1978) which modifies the position of hydroxyls in the ring structures of streptomycin and spectinomycin.

Therapeutic importance – Streptomycin is vestibulotoxic and cochleotoxic and has mostly been replaced by newer aminoglycosides. However, it is still sometimes used for specific purposes e.g., treatment of gonorrhea. Tuberculosis and brucellosis, and in combination with a beta-lactam agent or a glycopeptide for treating enterococcal endocarditis with high-level gentamicin (but not streptomycin) resistance. Streptomycin is also used as a pesticide in agriculture, although the known use is concentrated in the USA and Japan and is sparse in Europe.

Resistance occurrence – The *aad*A gene has been found in association with several transposons (Tn7, Tn21 etc.). Extrachromosomal elements (plasmids) carrying streptomycin resistance genes are common and can be found at high frequency in natural populations of bacteria (Shaw *et al.*, 1993) and in clinical isolates (Heym *et al.*, 1994). They are ubiquitous especially among gram-negative bacteria. In one study, 58.7% of the surveyed strains were shown to be streptomycin resistant and of these 55.5% carried the *ant*(3'')-Ia gene. Use of streptomycin or spectinomycin as a pesticide provides selective pressure in the environment, and will select for streptomycin resistant (plant-associated) bacteria. There are several recorded instances of such bacterial isolates (mostly obtained from apple orchards) in which streptomycin had been applied as a pesticide.

5.4. Ampicillin resistance: amp^r gene

Origin - The plasmid R7268, with its transposon Tn3 and the β-lactamase gene (*amp^r*, *bla*$_{(TEM-1)}$) was originally isolated from a hospital bacterium isolate [patient Thomas Edison Murphy (= TEM)] in 1963. A typical molecular cloning vector used for genetic engineering has a pBR322- or pUC-derived backbone. Such vectors contain the *bla* $_{(TEM-1)}$ gene of RSF 2124 plasmid.

Catalytic activity and substrate specificity - The *amp^r* gene encodes TEM-1 β-lactamase (Sanders and Sanders, 1992) which hydrolyses the amide bond in the beta-lactam ring of the antibiotic ampicillin. Substrates for the β-lactamase are ampicillin, penicillin G and amoxycillin. The TEM-1 enzyme has only a minor activity against recent cephalosporines and can be inhibited by β-lactamase inhibitors such as clavulanic acid or tazobactam. However, in the case of *E. coli*, a high expression rate of the β-lactamase may render the bacteria resistant to amoxicillin/tazobactam and other combinations of β-lactams with β-lactamase inhibitors (Sanders and Sanders, 1992). Moreover, mutations in the β-lactamase (e. g. TEM-30 to TEM-41) may result in reduced clavulanic-acid inhibition. In the classification scheme by Bush *et al.* (1995), such variants were introduced as a subclass of its own, 2br. So far, these inhibitor-resistant TEM β-lactamases (IRTs) have only been found in *E. coli* and sporadically in *Proteus mirabilis* or *Klebsiella* (Bermudes *et al.*, 1997).

Therapeutic importance – Ampicillin is an important antibiotic for humans as well as animals. In many cases, e.g. in the treatment of urinary tract infections, the use of ampicillin/amoxicillin is

recommended only when ampicillin/amoxicillin sensitivity has been proven. However, it is widely used to treat respiratory tract infections in humans. In the case of certain infections, e.g. with enterococci or *Listeria monocytogenes*, ampicillin is still considered to be the drug of choice. In veterinary medicine, ampicillin is used for treatment of bacterial infections in cattle, pigs and sheep and of mastitis in cattle. Amoxycillin is used for treatment of bacterial infections in cats and dogs, and respiratory and urogenital tract infections in cattle, pigs and sheep.

Resistance occurrence – There is a significant background of ampicillin-resistant bacteria in the normal human intestine. Of healthy humans, 19% harboured ampicillin-resistant *E. coli* in their intestine (DANMAP, 1997). Between 30 and 40% of Finns carry ampicillin-resistant coliform bacteria (Leistevuo *et al*., 1996). Because about 80% of these bacteria are *E. coli*, in which the most common resistance genes are of the TEM family, it is probable that about one third of Finns carry a TEM-1-containing bacterium. Also about 35% of *E. coli* isolates from clinical environments exhibit ampicillin resistance (Kresken *et al*., 1999; DANMAP, 2001). Around 90% of these cases are due to the β-lactamase type TEM 1 (Livermore, 1995). The corresponding gene is also widespread in other enterobacterial species as well as in *Haemophilus sp., Neisseria gonorrhoeae* and *Salmonella sp.*. The TEM-1 gene is common also in bacteria of animal origin. Danish data show that the prevalence of ampicillin resistant *E. coli* in broilers, cattle and pigs are 16, 0, and 10%, respectively (DANMAP, 2001). In clinical isolates, however, the occurrence of ampicillin resistance can be as high as 80% in cattle (DANMAP, 2001). During ampicillin therapy, the number of resistant bacteria increases because of their selective advantage in the intestine. Due to the extremely low probability of transfer of the resistance gene from genetically modified plants to intestinal bacteria, occurrence of such events would not add significantly to the existing background of ampicillin-resistant bacteria in the intestine.

5.5. Kanamycin resistance: *npt*III gene

Origin - The *npt*III [= *aph*AIII, *aph*(3')IIIa] gene originates from *Enterococcus faecalis* R plasmid.

Catalytic activity and substrate specificity – The gene encodes a type III aminoglycoside-3' phosphotransferase [APH(3')III] that is characterized by resistance to kanamycin, neomycin, paromomycin, ribostamycin, lividomycin, butirosin and gentamicin B (Shaw *et al*., 1993). Amikacin and isepamicin are also modified *in vitro*, although many strains express only a low level of resistance.

Therapeutic importance – Amikacin is a reserve antibiotic of significant value in the treatment of nosocomial infections involving Gram-negative organisms resistant to gentamicin and tobramicin.

Resistance occurrence – No systematically published information available.

5.6. Chloramphenicol resistance: Cm^R gene

Origin - The cm^R (= *cat)* gene originates from the transposon Tn9.

Catalytic activity and substrate specificity - The gene encodes chloramphenicol acetyltransferase (CAT) which catalyzes an acetyl-CoA-dependent acetylation of the antibiotic chloramphenicol, and thus, abolishes its antibacterial effect (Proctor and Rownd, 1982).

Therapeutic importance – Chloramphenicol is a broad-spectrum antibiotic. Its serious side-effect (aplastic anemia), although uncommon, restricts its systemic use in the developed world, where it is mainly used for topical treatment of eye, ear and skin infections in human and veterinary medicine. It is still used widely in developing countries. In humans, chloramphenicol is a first choice antibiotic for purulent meningitis of unknown etiology in patients who are highly allergic

to beta-lactam agents. Chloramphenicol is also an alternative for serious infections caused by bacteria resistant to other antibiotics. In veterinary medicine, chloramphenicol can be used for the treatment of serious infections in non-food-producing animals but is not authorised in the EU for use in food-producing animals.

Resistance occurrence – Resistant micro-organisms are widely found in the environment.

5.7. Tetracycline resistance: *tet*A gene

Origin - The *tet*A gene originates from the transposon Tn10.

Catalytic activity and substrate specificity - The gene encodes a membrane protein which causes the efflux of tetracyclines (Bryan, 1984). Tetracyclines are chemically closely related to one another, all being derivatives of naphthacene structure.

Therapeutic importance – Tetracycline and its derivatives are broad-spectrum antibiotics that have been extensively used in both human and veterinary medicine over more than 50 years for the treatment of a variety of infections. They are still used for treating infectious diseases due to organisms such as *Brucella, Chlamydia, Mycoplasma, Rickettsia and Vibrio* spp. etc and in the treatment of acne. However, due to widespread resistance their usefulness is now considerably more limited than earlier.

Resistance occurrence – The *tet* genes are widespread in the environment. The European project RESERVOIR in its final report (1999) records several instances of the widespread occurrence of diverse tet genes, as evidenced by molecular means.

6. Classification of antibiotic resistance genes by their biological distribution and based on the present state of therapeutic importance of the relevant antibiotics

If the transfer of an antibiotic resistance gene from the genome of a transgenic plant to that of a bacterium should occur at all, the risk associated with this very rare event should be viewed against the presence of antibiotic resistance genes in soil, plant, water and enteric bacteria. Furthermore, consideration must be given to the importance of specific antibiotics in therapeutic use. On the basis of these two criteria for evaluation, the above-mentioned antibiotic resistance genes useful as markers in genetic modification of plants have been assigned to three groups:

6.1. Group I

Group I contains antibiotic resistance genes which (a) are already widely distributed among soil and enteric bacteria and (b) confer resistance to antibiotics which have no or only minor therapeutic relevance in human medicine and only restricted use in defined areas of veterinary medicine. It is therefore extremely unlikely (if at all) that the presence of these antibiotic resistance genes in the genome of transgenic plants will change the already existing bulk spread of these antibiotic resistance genes in the environment or will impact significantly on human and animal health. This refers to the following two antibiotic resistance genes.

- ***npt*II gene**: The substrates of the APH(3´)II enzymes include the antibiotics, kanamycin, neomycin, paromycin, butirosin, gentamicin B and geneticin (G 418). The antibiotics of this category which are relevant for human therapy, amikacin, gentamicin (predominantly C_1, C_{1a} and C_2) and other aminoglycosides and aminocyclitoles, are not substrates for the APH(3´)-II enzymes. The *npt*II gene is widely spread in micro-organisms in the environment (Smalla *et al*., 1993; Leff *et al*., 1993).

- ***hph* gene**: Hygromycin is not used in human therapy, and there is no cross-resistance with other antibiotics used for human therapy. The antibiotic was originally developed for veterinary use and is still added in some parts of the world to animal feed as an anthelmintic.

6.2. Group II

Group II contains antibiotic resistance genes which (a) are widely distributed in micro-organisms in the environment (soil, plant, water and the mammal gut) and (b) confer resistance to antibiotics which are used for therapy in defined areas of human and veterinary medicine. The presence of these antibiotic resistance genes in the genome of transgenic plants will have only a minimal effect on the bulk spread of these antibiotic resistance genes in the environment, and therefore will have a minimal impact on human and animal health, if at all. Their presence in genetically modified plants will thus not contribute to their occurrence in bacteria. This refers to the following antibiotic resistance genes.

- ***Cm*^{R} gene**: Chloramphenicol-resistant micro-organisms are widely distributed in the environment, and many of these carry the *Cm*R gene. In the EU, chloramphenicol is rarely used for medical purposes because of the risk of causing aplastic anaemia and has not been authorized for use in food-producing animals.
- ***amp*^{r} gene:** It is reasonable to assume that almost every person on earth harbours or has harboured *Escherichia coli* cells containing the ampr gene in their intestinal tract, even without exposure to β-lactam antibiotics. This is supported by the observation that approximately 35 % of all clinical *E. coli* isolates are resistant to ampicillin (Kresken *et al*., 1999) of which 90%, in turn, are caused by TEM-1 β-lactamases (Livermore, 1995). Studies (BgVV, 1997) have also demonstrated that approximately 74 % of all *E. coli* isolates from cattle and swine are ampicillin resistant. Thus, even in the light of the clinical relevance of ampicillin, the presence of *amp*R (bla gene) in transgenes is not seen to siginificantly alter the existing pool of already resistant bacteria.
- ***aadA gene***: Streptomycin and spectinomycin are used in human medicine to a limited extent only (WHO, 1993). However, they still are of importance in human medicine for the treatment of tuberculosis (streptomycin) or gonorrhoea (spectinomycin). *Aad*A is to a limited extent prevalent in a range of environmental habitats (Van Overbeek *et al*., 2002).

6.3. Group III

Group III contains antibiotic resistance genes which confer resistance to antibiotics highly relevant for human therapy and, irrespective of considerations about the realistic value of the threat, should be avoided in the genome of transgenic plants to ensure the highest standard of preventive health care. This refers to the following antibiotic resistance genes.

- ***npt*III gene**: For use in human therapy, amikacin is an important reserve antibiotic whose therapeutic importance should not, even potentially, be reduced by the use of the *npt*III gene in the establishment of genetically modified plants.
- ***tet*A gene:** Tetracyclines are characterized by their wide spectrum of action and continue to be of therapeutic importance in human medicine; they are used to control *Brucella*, *Chlamydia*, *Mycoplasma*, *Rickettsia, Vibrio,* etc.

CONCLUSIONS AND RECOMMENDATIONS

With regard to current scientific and technical knowledge, ARMGs are still required in the majority of cases to ensure the efficient selection of transgenic events in plants. Also based on

present scientific knowledge, gene transfer from GM plants to bacteria under natural conditions cannot be excluded but it would be a very unlikely event. Therefore, such a rare event will not contribute effectively to the extant abundance of antibiotic resistance marker genes in bacteria in the environment (soil, plants, water and human and animal guts).

Directive 2001/18/EC states that the future development of genetically modified plants to be placed on the market and to be used in the production of food or feed should aim at avoiding genes which confer resistance to therapeutically relevant groups of antibiotics. With regard to this requirement the GMO Panel considered (1) the biosafety of using ARMGs in GM plants, (2) the extant pool of antibiotic resistance genes in natural bacteria and (3) best practices for the future use of ARMGs in GM plants.

The GMO Panel concludes that:

1. The frequency of horizontal gene transfer from GM plants to other organisms is very low for all three groups of ARMGs considered. This in itself is an important consideration with regard to the risk posed by the use of ARMGs.
2. For all of the antibiotics and resistances considered, it has been shown or is extremely likely that there is a considerable extant pool of resistance genes already present in the microbiota in the environment.
3. With regard to best practice, the requirements of Directive 2001/18/EC regarding therapeutically important antibiotics and the desire to limit the use of ARMGs, the Panel considers that ARMGs placed in group I (e.g the *npt*II marker) have a 13-year history of safe use in food crops. Furthermore, resistance to antibiotics in group I is widespread in naturally occuring prokaryotic gene pools. This, together with the other reasons provided in this document, indicates that there is no rationale for restricting or prohibiting the use of this group of ARMGs.

The use of ARMGs in group II should be restricted to field trial purposes and should not be present in GM plants to be placed on the market. Experimental releases of GM plants (according to part B of Directive 2001/18/EC) are generally confined, being limited in time and space. GM plants in experimental releases are not intended for use in foods or feeds. No hazardous effects on human health and the environment are thus to be expected from the presence of the ARMGs in GM plants used for experimental releases under approved conditions.

Given their current importance in clinical usage, the GMO Panel recommends that ARMGs placed in group III are not present in GM plants to be placed on the market or in plants used for experimental field trials (according to part B of Directive 2001/18/EC).

REFERENCES

Beever, D.E. and Kemp, C.F., 2000. Safety issues associated with the DNA in animal feed derived from genetically modified crops. A review of scientific and regulatory procedures. Nutr. Abstr. Rev.Series B: Livestock Feeds and Feeding, 70, 175-182.

Bennett, P. M., Livesey, C. T., Nathwani, D., Reeves, D. S., Saunders, J. R. and Wise, R., 2004. An assessment of the risks associated with the use of antibiotic resistance genes in genetically modified plants: report of the Working Party of the British Society for Antimicrobial Chemotherapy. J. Antimicrob. Chemother., 53, 418-431. http://jac.oupjournals.org/cgi/reprint/53/3/418.pdf

Bermudes, H., Jude, F. C. Arpin, C. Quentin, Morand, A. and Labia, R., 1997. Characterization of an inhibitor-resistant TEM (IRT) ß-lactamase in a novel strain of *Klebsiella pneumoniae*. Antimicrob. Agents Chemother., 41, 222

Bryan, L.E., 1984. In: Antimicrobial Drug Resistance, ed. Bryan, L.E. (Academic, New York), 241-277.

Bush, K., Jacoby, G.A. and Medeiros, A.A., 1995. A functional classification scheme for ß-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother., 39, 1211-1233.

Chambers, P.A., Duggan, P.S., Heritage, J. and Forbes, J.M., 2002. The fate of antibiotic resistance marker genes in transgenic plant feed material fed to chickens. J. Antimicrobial Chemotherapy, 49, 161-164.

Chen, I. and Dubnau, D., 2003. DNA transport during transformation. Frontiers in Bioscience, 8, 544-556.

Courvalin, P. and Carlier, C., 1981. Resistance towards aminoglycoside-aminocyclitol antibiotics in bacteria. J. Antimicrob. Chemother., 8, 57-69.

DANMAP, 1997. Danish Integrated Antimicrobial Resistance Monitoring and Research Programme. Consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark, Danish Zoonosis Institute, Danish Veterinary Institute, Copenhagen. http://www.dfvf.dk/Files/Filer/Zoonosecentret/Publikationer/Danmap/Danmap_1997.pdf

DANMAP, 2001. Danish Integrated Antimicrobial Resistance Monitoring and Research Programme. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark, Danish Zoonosis Institute, Danish Veterinary Institute, Copenhagen. ISSN 1600-2032. http://www.dfvf.dk/Files/Filer/Zoonosecentret/Publikationer/Danmap/Danmap_2001.pdf

Davies, J. and Smith, D.I., 1978. Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol., 32, 469–518.

De Vries, J., and Wackernagel, W,, 1998. Detection of *npt*II kanamycin resistance genes in genomes of transgenic plants by marker rescue transformation. Mol. and Gen. Genet., 257, 606-613.

De Vries, J., Meier, P., and Wackernagel, W., 2001. The natural transformation of the soil bacteria *Pseudomonas stutzeri* and *Acinetobacter* sp by transgenic plant DNA strictly depends on homologous sequences in the recipient cells. FEMS Microbiol Lett., 195, 211-215.

Duggan, P.S., Chambers, P.A., Heritage, J., and Forbes, J.M., 2000. Survival of free DNA encoding antibiotic resistance from transgenic maize and the transformation activity of DNA in ovine saliva, ovine rumen fluid and silage effluent. FEMS Microbiology Lett., 191, 71-77.

Duggan, P.S., Chambers P.A., Heritage, J. and Forbes, J.M., 2003. Fate of genetically modified maize DNA in the oral cavity and rumen of sheep. British J Nutrition, 89, 159-166.

EC, 2001. Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal of the European Communities, L106, 1-39. http://europa.eu.int/eur-lex/pri/en/oj/dat/2001/l_106/l_10620010417en00010038.pdf

Einspanier, R., Klotz, A., Kraft, J., Aulrich., K., Poser, R., Schwagele, F., Jahreis, G. and Flachowsky, G., 2001. The fate of forage plant DNA in farm animals: a collaborative case study investigating cattle and chicken fed recombinant plant material. European Food Research and Technology, 212, 129-134.

Fuchs, R.L., Heeren, R.A., Gustafson, M.E., Rogan, G.J., Bartnicki, D.E., Leimgruber, R.M., Finn, R.F., Hershman, A. and Berberich, S.A., 1993a. Purification and characterization of microbially expressed neomycin phosphotransferase II (NPTII) protein and its equivalence to the plant expressed protein. Bio/Technology, 11, 1537-1542.

Fuchs, R.L., Ream, J.E., Hammond, B.G., Naylor, M.W., Leimgruber, R.M. and Berberich, S.A., 1993b. Safety assessment of the neomycin phosphotransferase-II (NPTII) protein. Bio/Technology, 11, 1543-1547.

Garfinkel, D.J., Simpson, R.B., Ream, L.W., White, F.F., Gordon, M.P. and Nester, E.W., 1981. Genetic analysis of crown gall; fine structure map of the T-DNA by site-directed mutagenesis. Cell, 27, 143–153.

Gebhard. F. and Smalla. K,. 1998. Transformation of *Acinetobacter* sp. strain BD413 by transgenic sugar beet DNA. Applied and Environmental Microbiology, 64(4), 1550-1554. 261.

Gritz, L. and Davies, J., 1983. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene, 25, 179-188.

Heuer, H., Krögerrecklenfort, E., Egan, S., van Overbeek, L., Guillaume, G., Nikolakopoulou, T.L., Wellington, E.M.H., van Elsas, J.D., Collard, J.-M., Karagouni, A.D. and Smalla, K., 2002. Gentamicin resistance genes in environmental bacteria: prevalence and transfer. FEMS Microbiol. Ecol., 42, 289-302.

Heym, B., Honoré, N., Truffot-Pernot, C., Banerjee, A., Schurra, C., Jacobs, W.R., Jr., van Embden, J.D.A., Grosset, J.H. and Cole, S.T., 1994. Implications of multidrug resistance for the future of short-course chemotherapy of tuberculosis: a molecular study. Lancet 344, 293-298.

Hinton, M., Kaukas, A. and Linton, A.H., 1986. The ecology of drug resistance in enteric bacteria. J. Appl. Bacteriol. Symp. Suppl. 1986, 77S-92S.

Hohlweg, U. and Doerfler, W., 2001. On the fate of plant or other foreign genes upon the uptake in food or after intramuscular injection in mice. Mol. Gen. Genet., 265, 225-233.

Hollingshead, S. and Vapnek, D., 1985. Nucleotide sequence analysis of a gene encoding a streptomycin/spectinomycin adenyltransferase. Plasmid, 13, 17-30.

Kay, E., Vogel, T.M., Bertolla, F., Nalin, R. and Simonet P., 2002. *In situ* transfer of antibiotic resistance genes from transgenic (transplastomic) tobacco plants to bacteria. Appl. Environ. Microbiol., 68, 3345-3351.

Klein, J., Altenbuchter, J. and Mattes, R., 1998. Nucleic acid and protein elimination during the sugar manufacturing process of conventional and transgenic sugar beets. J. Biotechnology, 60, 145-153.

Klotz, A., and Einspanier, R, 1998. Nachweis von “Novel-Feed” im Tier? Beeintrachtigung des Verbrauchers von Fleish oder Milch ist nicht zu erwarten. Detection of “novel feed” in animals? Injury of consumers of meat or milk is not expected. Mais, 3, 109-111.

Kocabiyik, D. and Perlin, M.H., 1992. Altered substrate specificity by substitutions at Tyr218 in bacterial aminoglycoside 3’-phosphotransferase-II. FEMS Microbiol. Lett. 72, 619-624.

Kresken, M., Hafner, D. and von Rosenstiel, N., 1999. Zeitliche Entwicklung der Antibiotikaresistenz bei klinisch wichtigen Bakterienspecies in Mitteleuropa. Bundesgesundheitsblatt, 42, 17–25.

Kuhstoss, S., and Rao, R.N., 1983. Expression in *Streptomyces ambofaciens* of an *Escherichia coli* K-12 gene which confers resistance to hygromycin B. Gene 26(2-3), 295-9.

Leboul, J., Davies, J., 1982. Enzymatic modification of hygromycin B in *Streptomyces hygroscopicus*. J Antibiot., 35(4), 527-528.

Leff, L. G., Dana, J. R., McArthur, J. V. and Shimkets, L. J., 1993. Detection of Tn5-like sequences in kanamycin-resistant stream bacteria and environmental DNA. Appl. Environ. Microbiol., 59, 417-421.

Leistevuo, T., Leistevuo, J., Österblad, M. *et al*., 1996. Antimicrobial resistance of fecal aerobic gram-negative bacilli in different age groups in community. Antimicrob. Agents Chemother., 40, 1931-1934.

Lewin, B., 2000. Genes VII. Oxford University Press, Oxford, UK.

Livermore, D. M., 1995. ß-lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev., 8, 557-584.

Lorenz, M.G., and Wackernagel, W., 1994. Bacterial gene transfer by natural genetic transformation in the environment. *Microbiological Reviews*, 58, 563-602.

Maliga, P., Svab, Z., Harper, E. C. and Jones J. D. G., 1988. Improved expression of streptomycin resistance in plants due to a deletion in the streptomycin phosphotransferase coding sequence. Mol. Gen. Genet., 214, 456-459.

Malpartida, F., Zalacain, M., Jimenen, A. and Davies, J., 1983. Molecular cloning and expression in *Streptomyces lividans* of a hygromycin B phosphotransferase gene from *Streptomyces hygroscopicus*. Biochem Biophys Res Commun, 117, 6-12.

Martin-Orue, S.M., O'Donnell, A.G., Arino, J., Netherwood, T., Gilbert H.J. and Mathers J.C., 2002. Degradation of transgenic DNA from genetically modified soya and maize in human intestinal simulations. British Journal of Nutrition, 87, 533-542.

Mercer, D.K., Scott, K.P., Bruce-Johnson, W.A., Glover, L.A. and Flint, H.J., 1999. Fate of free DNA and transformation of the oral bacterium *Streptococcus gordonii* DL1 by plasmid DNA in human saliva. Applied and Environmental Microbiology, 65, 6-10.

Mercer, D.K., Scott, K.P., Melville, C.M., Glover, L.A. and Flint, H.J., 2001. Transformation of an oral bacterium via chromosomal integration of free DNA in the presence of human saliva. FEMS Microbiology Letters, 200, 163-167.

Nap, J.P., Bijvoet, J. and Stikema, W.J., 1992. Biosafety of kanamycin-resistant transgenic plants: an overview. Transgenic Res., 1, 239-249.

Netherwood, T., Martin-Orue, S.M., O'Donnell, A.G., Gockling, S., Gilbert, H. and Mathers, J., 2002. Transgenes in genetically modified soya survive passage through the human small bowel but are completely degraded in the colon. In: Technical report on FSA project 'Evaluating the risks associated with using GMOs in human foods', 20-31. http://www.food.gov.uk/multimedia/pdfs/gmnewcastlereport.PDF

Nielsen, K.M. , Bones, A.M., Smalla, K. and Van Elsas, J.D., 1998. Horizontal gene transfer from plants to terrestrial bacteria – A rare event? FEMS Microbiol. Rev., 22, 79-103.

Nielsen, K.M. , Van Elsas, J.D. and Smalla, K., 2000. Transformation of Acinetobacter sp. strain BD413 (pFG4deltanptII) with transgenic plant DNA in soil microcosms and effects of kanamycin on selection of transformants. Appl. Environ. Microbiol., 66, 1237-1242.

Perlin, M.H., and Lerner, S.A., 1986. High-level amikacin resistance in Escherichia coli due to phosphorylation and impaired aminoglycoside uptake. Antimicrob Agents Chemother., 29, 216-24

Proctor, G.N. and Rownd, R.H., 1982. Rosanilins: indicator dyes for chloramphenicol-resistant enterobacteria containing chloramphenicol acetyltransferase. J. Bacteriol., 150, 1375–1382.

Rao,R.N., Allen, N.E., Hobbs, J.N., Alborn, W.,E., Kirst, H.A., and Paschal, J.W., 1983. Genetic and enzymatic basis of hygromycin B resistance in *Escherichia coli*. Antimicro. Agents Chemother., 24, 689-695.

Redenbaugh, K., Berner, T., Emlay, D., Frankos, B., Hiatt, W., Houck, C., Kramer, M., Malyj, L., Martineau, B., Rachman, N., Rudenko, L., Sanders, R., Sheehy, R. and Wixtrom, R., 1993. Regulatory issues for commercialization of tomatoes with an antisense polygalacturonase gene. In Vitro Cell. Dev. Biol., 29P, 17-26.

Redenbaugh, K., Hiatt, W., Martineau, B., Lindemann, J. and Emlay, D., 1994. Aminoglycoside 3'-phosphotransferase II (APH(3')II): Review of its safety and use in the production of genetically engineered plants. Food Biotechnol., 8, 137-165.

Salyers, A., 1996. The real threat from antibiotics. Nature, 384, 304.

Sanders, C. and Sanders, W.E., 1992. β-Lactam resistance in gram-negative bacteria: global trends and clinical impact. Clin. Infect. Dis., 15, 824–839.

Schlüter, K., Fütterer, J., Potrykus, I., 1995. Horizontal. gene transfer from a transgenic potato line to a bacterial pathogen *(Erwinia chrysanthem*i) occurs if at all at an extremely low frequency. Biotechnology, 13, 1094-1098.

Schmitz, F.J., Fluit, A.C., Gondolf, M., Beyrau, R., Lindenlauf, E., Verhoef, J., Heinz, H.P., Jones, M.E., 1999). The prevalence of aminoglycoside resistance and corresponding resistance genes in clinical isolates of staphylococce from 19 European hospitals. J. Antimicrob Chemother., 43, 253-259

Schubbert, R., Hohlweg, U., Renz, D. and Doerfler, W., 1998. On the fate of orally ingested foreign DNA in mice: chromosomal association and placental transmission to the fetus. Mol. Gen. Genet., 259, 569-576.

Schubbert, R., Lettmann, C. and Doerfler, W., 1994. Ingested foreign (phage M13) DNA survives transiently in the gastrointestinal tract and enters the bloodstream of mice. Mol. Gen. Genet., 242, 495-504.

Schubbert, R., Renz, D., Schmitz, B. and Doerfler, W., 1997. Foreign (M13) DNA ingested by mice reaches peripheral leukocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA. Proceedings of the National Academy of Science USA, 94, 961-966.

Shaw, K. J., Rather, P. N., Hare, R. S. and Miller, G. H., 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol. Rev., 57, 138-163.

Smalla, K, Van Overbeek, L.S., Pukall, R. and Van Elsas, J.D., 1993. Prevalence of *npt*II and Tn5 in kanamycin-resistant bacteria from different environments. FEMS Microbiol. Ecol. 13, 47-58.

Smalla, K. and van Elsas, J. D., 1996. Monitoring genetically modified organisms and their recombinant DNA in soil environments. In: J. Tomiuk, K. Wöhrmann and A. Sentker (Eds). Transgenic Organisms - Biological and Social Implications. Birkhäuser Verlag, Basel, Switzerland, 127-146.

Svab, Z., Harper, E. C., Jones, J. D. G. and Maliga, P., 1990. Aminoglycoside-3''-adenyltransferase confers resistance to streptomycin and spectinomycin in *Nicotiana tabacum*. Plant Mol. Biol., 14, 197-205.

Tomalsky, M.E. and Crosa, J.H., 1987. Tn1331, a novel multiresistant transposon encoding resistance to amikacin and ampicillin in *Klebsiella pneumoniae*. Antimicrob. Agents Chemother., 31, 1955-1960.

Van Overbeek, L.S., Wellington, E.M.H., Egan, S., Smalla, K., Heuer, H., Collard, J.-M. ,Guillaume, G., Karagouni, A., Nikolakopoulou, T.L., and van Elsas, J.D., 2002. Prevalence of streptomycin resistance genes in bacterial populations in European habitats. FEMS Microbiol Ecol., 42, 277-288.

Waldron, C., Murphy, E. B., Roberts, J. L., Gustafson, G. D., Armour, S. L. and Malcom, S. K., 1985. Resistance to hygromycin B — a new marker for plant transformation studies. Plant Mol. Biol., 5, 103-108.

SCIENTIFIC PANEL MEMBERS

Hans Christer Andersson, Detlef Bartsch, Hans-Joerg Buhk, Howard Davies, Marc De Loose, Michael Gasson, Niels Hendriksen, Colin Hill, Sirpa Kärenlampi, Ilona Kryspin-Sørensen, Harry Kuiper, Marco Nuti, Fergal O'Gara, Pere Puigdomenech, George Sakellaris, Joachim Schiemann, Willem Seinen, Angela Sessitsch, Jeremy Sweet, Jan Dirk van Elsas and Jean-Michel Wal

ACKNOWLEDGEMENT

The GMO Panel wishes to thank Armine Sefton for her contribution to the draft opinion.