Antibiotic resistance markers in genetically modified plants: a risk to human health?

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Cotransformation with an antibiotic-resistance marker is often necessary in the process of creating a genetically modified (GM) plant. Concern has been expressed that the release of these markers in GM plants may result in an increase in the rate of antibiotic resistance in human pathogens. For such an event to occur, DNA must not be totally degraded in field conditions, and the antibiotic-resistance marker must encounter potential recipient bacteria and be taken up by them, before being integrated into the bacterial genome, and the genes then expressed. In addition, the new recombinant must overcome the physiological disadvantage of acquisition of a piece of foreign DNA, probably in conditions where the new gene does not provide a selective advantage. We review each of these stages, summarising the investigations that have followed each of these steps. We contrast the potential increase in the antibiotic resistance reservoir created by antibiotic-resistance markers in GM plants with the current situation created by medical antibiotic prescribing. We conclude that, although fragments of DNA large enough to contain an antibiotic-resistance gene may survive in the environment, the barriers to transfer, incorporation, and transmission are so substantial that any contribution to antibiotic resistance made by GM plants must be overwhelmed by the contribution made by antibiotic prescription in clinical practice.

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Introduction

Genetic modification in plants involves the transfer of one or more selected genes into recipient cells by a variety of processes, most commonly using *Agrobacterium tumefaciens*, or a direct gene transfer alternative (eg, particle delivery). The frequency of such transfers in plants is low and unless transformation results in the acquisition of a selective phenotype, the isolation of clones from the background of untransformed cells is difficult to achieve. Therefore, the genes to be transferred are cotransformed with a marker that enables the desired phenotype to be recovered. The most frequently used selectable marker in plant cell modification is the *nptII* gene, which encodes a neomycin phosphotransferase, an enzyme that inactivates the aminoglycoside antibiotics neomycin, kanamycin, and paromomycin. When integrated in the plant genome as a chimeric construct that includes a plant promoter, the *nptII* open reading frame, and an appropriate 3′ non-coding sequence (transcriptional terminator and polyadenylation sequence), the protein is produced, conferring resistance to concentrations of kanamycin that are lethal for non-transformed cells. Thus cells cotransformed by *nptII* and a gene of interest can be selected on a kanamycin-enriched growth medium, facilitating the identification of genetically modified (GM) plants.

The issue of the safety of incorporating antibiotic-resistance markers into GM plants has been a matter of public debate since the early stages of their development. As part of the safety assessment of GM plants, a number of expert committees have examined whether the *nptII* gene in Calgene's FlavrSavr GM tomato or the ampicillin resistance marker (*amp*^{r} [beta-lactamase *bla* TEM1]) in Novartis's Bt176 maize could be transferred from GM plants back to bacteria, thus becoming an additional source of antibiotic-resistant pathogens.

The UK Advisory Committee for Novel Food and Processes voiced concerns about the presence of an ampicillin resistance gene in Novartis's Bt176 maize and recommended that the UK should vote against the EU approval of the product. This recommendation paved the way for a precautionary approach that continues to prevail. The scientific committees of the EU Commission, the Scientific Committee on Animal Nutrition, and the Scientific Committee on Food subsequently concluded that the *amp*^{r} gene inserted in Novartis Bt176 maize did not present any risk for the health of human beings or animals. In 1998 the EU Commission permitted the marketing of GM glufosinate-resistant rapeseed that included *nptII* (Commission Decision 98/291/EC), their experts having concluded that the *nptII* marker was safe in this application. A number of consultations were held at international and national levels as well as within scientific societies. These consultations resulted in the publication of a number of scientific papers and reports.[1–8] Further reports from international organisations and governmental bodies have been published.[9–16]

Many of the papers that assess the medical, ecological, economic, and societal value/risk of antibiotic-resistance markers actually end with conclusions that leave some lingering doubt about the safety of the technology. For example, Smalla and colleagues[17] wrote: "Given the fact that antibiotic resistance genes, often located on mobile genetic elements are already widespread in bacterial populations and that HGT [horizontal gene transfer] events from transgenic plants to bacteria are supposed to occur at extremely low frequencies and have not 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".

This is an example of the fact that most scientists involved in this area have concluded that the presence of antibiotic-resistant marker genes in plants does not include any risk to human or animal health, although there are some exceptions.[18] The public, however, remain sceptical, assisted by lurid headlines in popular newspapers warning of the danger of "Frankenstein foods". Beyond the polemic of opponents and the enthusiasm of supporters it is necessary to carefully consider the biological barriers to the transfer of antibiotic-resistance markers into human pathogens and the possible clinical consequences of this transfer. We will evaluate the scientific evidence regarding the safety of antibiotic-resistance markers for human health. We will focus on the *nptII* gene, which is the most frequently used antibiotic-resistance marker, involved in one-fifth of the 1431 applications of GM plant field tests submitted in the USA between Jan 2003 and March 2004.

Transfer of antibiotic-resistance marker genes in the natural environment

To evaluate the risk potentially caused by the transfer of antibiotic-resistance markers to animal and human pathogens it is necessary to understand the process that would have to occur and the barriers that would have to be overcome. The genes present in a GM crop would have to be transferred from plant material to bacteria through random transformation and recombination. This would probably occur in an environment that is not optimal for transformation. Finally, the acquisition of the antibiotic-resistance marker may impose a physiological cost on the transformed bacteria, imposing a negative selection pressure in the absence of positive antibiotic selection. Each of these issues will be explored in detail.

Gene transfer

The following conditions must be fulfilled for an antibiotic-resistance marker to be transferred in to a new bacterial host. First, upon lysis of the plant cells, DNA must not be totally degraded, then there must be a physical encounter between DNA fragments of an appropriate size with recipient bacteria. DNA must then be taken up by the bacteria, and become integrated in the bacterial genome. Finally, the genes must be expressed.

The fate of linear plant DNA fragments released in agricultural fields

Some plant residues (eg, roots, stems, leaves, and pollen grains) remain in the field after harvest. This remaining plant material is often ploughed into the soil to decay. The destruction of the plants cells by mechanical or chemical degradation or by microbial action, results in the release or digestion, or both, of their cellular contents, including DNA. Despite multiple factors contributing to the degradation of the plant DNA—including the nucleases of the plant itself, nucleases released by surrounding microbes, and mechanical shearing—intact DNA fragments can be found in the environment. The protection of DNA against nuclease attack is shown to occur in soil[19–22] and aquatic environments.[23] The end result is that rare fragments large enough to include an open reading frame of the size of an antibiotic-resistance marker gene can persist long enough in these apparently hostile environments to allow the potential uptake and incorporation into bacteria.

The case of silage

Silage provides an important means of storing forage crops. Whole plants are harvested before maturity, chopped, and stored under anaerobic conditions. The ensiled material undergoes a lactic fermentation, resulting in a rapid drop in pH below 5, preventing the further growth of other bacteria and preserving the forage for the whole season. A study by Novartis (EU application C/F/94/11-03) demonstrated that DNA in ensiled maize is substantially degraded, probably due to the low pH. Duggan and colleagues[24] used *Escherichia coli* as receptor cells to demonstrate that the transforming ability of plasmid DNA disappeared after less than 2 minutes of incubation with silage fluid, even though rather long stretches of DNA could be amplified by PCR following a 30 minute incubation in the same conditions.

The fate of DNA in the gastrointestinal tract of vertebrates

Until recently, the fate of DNA in the gastrointestinal tract had not been extensively investigated, probably because there was a generally held dogma that food DNA could not resist low stomach pH and degradation by pancreatic DNase. Schubbert and colleagues[25,26] showed that plasmid and bacteriophage DNA introduced into mice by oral gavage at very high concentrations was not totally degraded in the intestinal tract. Fragments of DNA of hundreds to thousands of basepairs, enough to include the open reading frame of an antibiotic resistance gene, were found in the intestine of the mice. Although surviving DNA represented less than 4% of that ingested, this was still a substantial amount of genetic material available for transfer to gut bacteria. The authors did not find the ingested DNA sequences in either intestinal bacteria or in phage particles, indicating that no transformation had occurred in culturable bacteria from the digestive tract of the mice. A number of investigations have used PCR to follow the fate of feed DNA in the gastrointestinal tracts of animals.[27–29] These studies reached similar conclusions—a small proportion of DNA in fragments of the size of an antibiotic-resistance marker can resist degradation in the gastrointestinal tract. Not surprisingly, the level of degradation was found to increase as the feed moved along the gastrointestinal tract.

Netherwood and co-workers[30] did the first study of the fate of the DNA of transgenic soybeans in human

volunteers. They showed that a variable proportion of a 180 bp fragment of a transgene, the *epsps* gene, is still amplifiable by PCR after passage through the intestine of patients with ileostomies. A PCR designed to amplify only the full length sequence genes was able to detect intact genes in individuals in whom at least 10^4 copies of the 180 bp fragment had survived. However, the authors suggest that this result should be treated with caution, since no *epsps* sequence could be detected in the faeces of volunteers with an intact large gut. Curiously, a few of the ileostomised patients had evidence of the short length fragment of the *epsps* gene incorporated into bacteria of their flora before the experiments started, but no full length genes. It proved impossible to culture and identify any of the species harbouring partial transgenes. In a study of survival of transgenes in ruminant animals, persistence patterns of endogenous and recombinant *epsps* rapeseed DNA were inversely related to substrate digestibility, but did not differ between parental and GM rapeseed, nor among fragments.[31] Detection of fragments was representative of persistence of the whole transgene. No *epsps* fragments were amplifiable in microbial DNA, suggesting that transformation had not occurred during the experiment. The authors conclude that the uptake of transgenic DNA fragments by ruminal bacteria is probably precluded or time-limited by rapid degradation of plant DNA upon plant cell lysis.[31] A similar study in sheep showed that digestion of plant material and release of transgenic DNA can occur in the ovine small intestine but free DNA was rapidly degraded at neutral pH.[32]

Bacterial transformation in natural environments

None of the investigations cited above were able to demonstrate any natural transfer of an antibiotic-resistance marker to the relevant surrounding bacteria. To date, less than 50 bacterial species are known to be transformable,[20,33] although new species will certainly be added to the existing list. Competence is difficult to achieve under laboratory conditions but it is nevertheless known to occur in natural environments. For example, the seminal paper by Griffiths[34] showed that transformation of *Streptococcus pneumoniae* happened in the blood stream of a mouse. Also, Bertolla and colleagues[33] showed that *Ralstonia solanacearum* can spontaneously achieve the competent stage in plants; this is also the case for *Acinetobacter* spp.[35] Some bacterial species do not develop the competent state in nature, although they can be transformed following specific laboratory treatments that allow extracellular DNA to penetrate the cell membranes. However, it is possible that conditions more favourable to genetic exchange in the natural environment could occur, as is the case for the common intestinal bacterium *E coli*.

DNA uptake

The uptake of DNA by a competent cell requires, above all, the physical encounter of the cell with the transforming DNA. Uptake is defined as the transition of bacteria-bound DNA into a DNase resistant state.[19,36] It should be noted that in some genera (eg, *Haemophilus influenzae* and *Neisseria* spp) DNA uptake involves the recognition of specific sequences of nucleotides, thus preventing the uptake of foreign genetic material. In most other cases, including *Bacillus subtilis* and *S pneumoniae*, DNA of any origin can be taken up. Competition takes place between all of the DNA fragments available with those bearing antibiotic-resistance markers, which represent approximately one-millionth of the plant genome. This competition, together with the additional contribution of DNA of decaying microbes, means that antibiotic-resistance markers are only a small proportion of the overall DNA available for transformation.[37]

Stabilisation and expression of the incoming genes

Incorporating a linear plant DNA fragment into either the cell chromosome or a transmissible plasmid is the next critical step in the transformation process. It involves the integration of an incoming DNA fragment into the chromosome, or a resident plasmid. Such processes are known to be extremely well controlled in bacteria and mediated either through homologies or transposition capacities.

S pneumoniae is a model for the understanding of the role of DNA-mediated transformation in the evolution of the genome.[38] The presence of sequence homology between the donor DNA and the recipient chromosome appears to be a strict requirement for the integration of foreign DNA into the chromosome of *S pneumoniae*;[39] this is also true in other transformable bacteria. De Vries and co-workers[40–42] designed plasmid and genomic constructs containing a partly deleted *nptII* gene and inserted those into the transformable strain *Acinetobacter* sp BD413. The original strain cannot be transformed to kanamycin resistance at a measurable rate by *nptII* genes present in transgenic plant DNA.[40–43] However, the engineered strain including the partial deletion can take up the *nptII* antibiotic-resistance marker from potato and beet DNA under laboratory conditions. De Vries and Wackernagel[42] showed that homology is required at least on one end of the DNA fragment to act as an anchor, allowing illegitimate recombination to take place on the other end of the fragment. The rate of transformation in this case was 10^{-9} lower than when the donor sequence matches the sequence of the recipient DNA at each end. None of the investigations cited above were able to demonstrate any natural transfer of genes liberated in those diverse environments into the surrounding microflora. Other investigations by Smalla and colleagues[4] and by Bertolla and co-workers[33] failed to detect any transfer of antibiotic-resistance marker genes into bacteria from soil where transgenic sugar beet or tobacco have been grown, although the DNA including the genes could be

isolated from the soil samples. Kay and co-workers[44] could not detect any transfer in plants of *nptII* in transgenic tomatoes coinfected with the transformable acinetobacter strain and *Ralstonia solanacearum*. Finally, once integrated, the antibiotic-resistance marker gene would have to be sufficiently expressed for the host bacterium to be resistant to the antimicrobial agent. For sufficient expression to occur, the *nptII* antibiotic-resistance marker associated with a plant promoter would have to be put under the control of a bacterial promoter during the act of insertion to ensure expression of the kanamycin-resistance gene. This requirement is by contrast with the active transfer of the genes generated by antibiotic selection in the next section.

Resistance reservoirs

The bacteria to which transfer is most likely to occur because of genetic factors, transformability, and ability to incorporate divergent DNA may not themselves be pathogens. The likelihood of the antibiotic-resistance marker being transferred to human pathogens from the environment or organisms in animals depends on the connection between these species. This idea is encompassed in the concept of resistance reservoirs[45] (figure).

Courvalin[18] and Summers[46] both emphasise the fact that the propagation of resistance is an ecological problem, meaning that the spread of antibiotic resistance involves the whole environment. Quoting O'Brien: "... there is growing evidence ... that antimicrobial resistance genes and their genetic vectors, once evolved in bacteria of any kind, anywhere, can spread indirectly through the world's interconnecting commensal, environmental, and pathogenic bacterial populations to other kinds of bacteria anywhere else".[47] How well resistance mechanisms spread depends on the frequency and diversity of the resistance mechanisms emerging, the number of connections between populations, and the ease of transmission between niches. The concept of reservoirs of antibiotic resistance is based on this ecological view. Thus, the concern about antibiotic-resistance markers including *nptII* is that the addition of a large number of antibiotic-resistance marker genes released as DNA fragments into the environment will substantially alter the size of the resistance reservoirs, therefore making it more likely that pathogens become resistant to kanamycin and neomycin.

Emergence of resistance

Resistance to an antimicrobial, as a phenotype, may arise through changes to a number of physiological mechanisms—eg, the chemical modification of the biological target, the inactivation of permeases, and the activation of efflux mechanisms. Many different genes can be involved in these processes. For example, one study identified not less than 22 different genetic mechanisms involved in the inactivation of aminoglycosides.[48] The broader the genetic diversity of resistance mechanisms, the more effective the colonisation potential for resistance in a given niche or between niches. For those antibiotics for which chromosomal mutation is the mechanism, the presence of antibiotic is essential for resistance to develop. Further spread is facilitated by an environment where antibiotics are present.

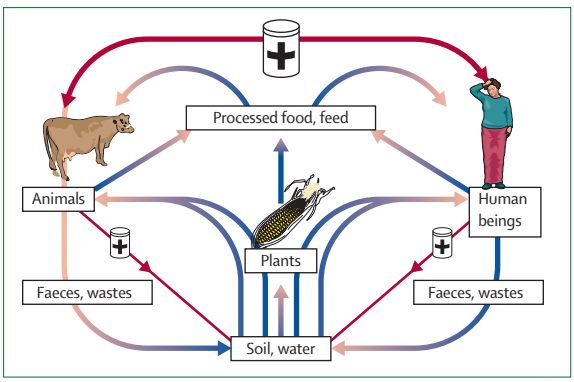

Figure: **The dissemination of bacteria and bacterial genes in our environment**
Dark arrows indicate a flux of bacteria and/or bacterial genes between the different biotopes. Light grey arrows show where the selection is exerted by the use of antibiotics. Antibiotics are involved mainly in therapeutic use in human beings and in animals and in animal husbandry. Substantial amounts of antibiotics are eliminated in faeces or urine, or are included in animal feed, and can reach ground waters and soil, where they may exert an unintended selective pressure in sewage, soil, or ground water. Although various species have become highly specialised and grow preferentially in unique habitats, human and animal activities result in an intermingling of bacterial species. These mixtures allow limited exchanges between various biotopes/reservoirs but they are quite strongly counter-selected due to the physiological requirements of the various species of bacteria. Therefore potential genetic exchanges are quite rare.

Selection

Antibiotics eliminate both pathogens and commensal organisms that are susceptible. By removing commensal organisms, a niche is created that is ready for invasion by resistant bacteria, whether they are primary pathogens or organisms of low virulence. Thus, antibiotics have a double effect in encouraging resistance—providing the initial selection pressure that favours their emergence and providing an ecological niche for resistant strains to colonise. This double effect is seen most clearly in the hospital environment where patients are prone to colonisation with resistant organisms after just a few days in hospital. Extended treatment with antibiotics can cause a substantial alteration in the normal flora of the host, which may permit an overgrowth of organisms that are naturally resistant, seen in its simplest form in patients who develop oral thrush during antibiotic treatment. More subtly, long-term low-dose treatment is most likely to result in selection of resistance, as has been demonstrated in the case of beta-lactam resistance in

S pneumoniae.[49,50] Resistance emerges through transformation of the organism by small sequences from closely related species that inhabit the same ecological niche. This transformation occurs at a very low rate and the organisms survive when a selective pressure is applied. The evidence for this is that penicillin-binding protein genes of sensitive strains are highly conserved (less than 3% divergence) whereas those of resistant strains diverge by more than 20%.[51] Similar effects can occur in food animals prescribed antibiotic growth promoters. The selective environment created allows the expansion of organisms such as vancomycin-resistant enterococci that may then be passed into the human population through the food chain. This effect can continue after the antibiotic is withdrawn from use.[52]

Mutation

Mutation has had a major role in the development of *bla* TEM alleles encoding beta-lactamases with an extended spectrum following the stepwise introduction of new beta-lactam agents.[53,54] The situation is different for most other antibiotic resistance genes, especially *nptII*. A single mutation in *nptII* could result in an alteration of the specificity of the enzyme neomycin phosphotransferase II, making it able to phosphorylate amikacin in vitro.[55] However, it should be noted that the authors were unable to obtain a clinically significant change in resistance by introducing a single mutation in the *nptII* gene. Significant resistance could only be obtained under laboratory conditions, as a consequence of two simultaneous rare mutations affecting two different genes—*nptII* and a gene encoding a permease.[56] The natural occurrence of such double mutants has not been reported, although resistance to gentamicin, amikacin, and tobramycin caused by the presence of a number of other antibiotic resistance genes is unfortunately widespread.[57]

Transmission of resistance genes

Resistance gene association and clustering

The carriage of multiple drug resistance determinants is the rule rather than the exception. Multiple resistance often involves clustered resistance genes having been recruited on transmissible elements such as transposons, plasmids, and integrons.[58] Well-established clones of resistant bacteria are able to enhance their resistance phenotypic portfolio by acquiring new resistance genes. The organisms equipped with multidrug resistance are highly resistant to the loss of any of these resistance mechanisms because exposure to any one of the markers carried will select for the clone. Not only are resistant bacterial clones selected, but also their gene capture devices. Thus, we must think beyond the individual organisms and consider the transmissible genetic elements that they possess and their population genetics. Consideration of these issues is especially important when one thinks of the hospital environment where bacteria are exposed to a multiplicity of antibiotic agents and selection pressure is at its highest.[58] Even where organisms do not develop resistance as a result of transmissible genetic elements, similar forces are exerted. In *Mycobacterium tuberculosis*, resistance emerges through point mutations in chromosomal genes[59] but once resistance emerges to one antibiotic the risk of subsequent resistance emerging is substantially higher.[60]

Horizontal gene transfer

Bacteria are capable of transferring genes to other microbial species present in the same niche. Recent studies have indicated that we do not have methods to detect such transfer in a field environment.[61] This process can occur by complex DNA structures dedicated to gene dissemination—eg, plasmids associated with specific transmission mechanisms.[62] It is noteworthy that, besides the literature specifically dedicated to the risk assessment of GM plants, most scientific papers dedicated to antibiotic resistance do not even mention random transformation with linear segments of DNA as an important contributor to newly acquired resistance. Conjugation between compatible mating types of bacteria has been proven to be by far the major cause of the horizontal dissemination of resistance happening in most natural habitats[4,7,47,63] at frequencies that can reach 3% of the cells of a recipient strain in the avian gastrointestinal tract[64] and up to 1% of bacteria in plants.[44] The most frequent neomycin/kanamycin resistance genes are plasmid-borne[48] and are thus easily transferred by conjugation.

Clonal/vertical gene dissemination

Once a resistance gene has been transferred horizontally to a given species with successful integration of the gene into a replication competent unit (chromosome or plasmid), resistant bacteria multiply as much as allowed by the surrounding ecosystem. Since the direct contribution of conjugation to the frequency of resistant bacteria is at most of the order of 10^{-5}, it is clonal multiplication that allows the resistant bacteria to invade a given niche.[65] As shown above, in most environments including soils, animals, and human beings, the frequencies of neomycin/kanamycin-resistant bacteria, including pathogens, are nowadays in the order of a few percent.[65,66] Such frequencies are the result of clonal multiplication of resistant bacteria. Thus, when resistant populations are established, the withdrawal of selective agents—eg, the antibiotic—does not necessarily result in a rapid return to the initial resistance frequency.[8]

Migration and contamination

Resistant bacteria are transported between niches by various routes (figure). The appearance of a new strain with a substantial biological advantage—eg, antibiotic

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resistance—can result in an epidemic, as evidenced by the international spread of meticillin-resistant *Staphylococcus aureus* and multidrug-resistant *Acinetobacter baumanii*. Such migration is especially important in the hospital environment when large numbers of individuals who are vulnerable to infection are housed together and often subject to antibiotic treatment that provides a substantial selection advantage to resistant pathogens.[67] Transfer of a resistant organism from an environmental reservoir to this nosocomial environment can be particularly dangerous.

The physiological price of resistance gene acquisition

There is a widespread belief that organisms pay a substantial physiological price for the acquisition of a resistance determinant; most commonly, it is supposed that this price includes a decrease in virulence. This effect in human pathogens was first suggested by Middlebrook and Cohn,[68] who demonstrated that *M tuberculosis* resistant to isoniazid appeared less pathogenic in a guineapig model. More recently, support for the concept of a physiological "price-tag" for resistance acquisition was found in an elegant series of experiments investigating resistance in *E coli*.[69] Numerous studies have indeed shown that resistant genotypes are less fit than their sensitive counterparts in the absence of antibiotic, indicating a cost of resistance.[70] However, there is an important caveat: these studies have put resistance genes into naive bacteria that have no evolutionary history of association with the resistance genes. Therefore, an important question is whether bacteria can overcome the cost of resistance by evolving adaptations that counteract the harmful side-effects of resistance genes. In fact, several experiments, both in vitro and in vivo, show that the cost of antibiotic resistance can be substantially diminished, even eliminated, by evolutionary changes in bacteria over rather short periods of time.[71] As a consequence, it becomes increasingly difficult to eliminate resistant genotypes simply by suspending the use of antibiotics.[72] Most of these experiments have been done with the transfer of a plasmid, a situation that resembles the putative transfer of an antibiotic-resistance marker from GM plants. Similar results were obtained when the genetic change is smaller—ie, when there is a single point mutation.[73] *E coli* that have become resistant to streptomycin through chromosomal mutation have a substantial reduction in their growth rate that could be compensated by mutation at other sites.

We have previously demonstrated that the in-vitro selection of rifampicin resistance in *M tuberculosis* gives rise to a limited repertoire of mutations, most of which are associated with a reduction in fitness, as measured by comparative growth velocity.[74] The relative fitness of each of these mutations was substantially correlated with the frequency of clinical isolation in practice,[74] implying that once resistance has emerged it is the relative fitness of the organism that decides whether it will survive in the competitive natural environment. These findings have been confirmed in *M tuberculosis*;[75] similar results have been observed for *rpoB* mutations in *S aureus*[76] and recently in *Mycobacterium smegmatis*,[77] *M tuberculosis*,[78] and *Salmonella typhimurium*.[79] However, we must be cautious to generalise these results. The fitness deficit has not been universally demonstrated. In experiments using *S pneumoniae* we were unable to show a substantial fitness deficit when strains became resistance to fluoroquinolones through point mutation.[80] Additionally, once organisms have developed resistance and survived, there is adaptation to the fitness deficit, as demonstrated in vitro in longitudinal transfer studies of up to 10 000 generations[81,82] but more importantly in the transfer of organisms between individuals during a hospital outbreak.[83] From this, we may predict that the transfer of a whole gene from a GM plant to human or animal bacteria is likely to impose a substantial fitness deficit that would not be compensated by other genes transferred at the same time. However, should the transfer take place and the modified organism survive for any period, adaptation will take place and the transfer fixed in the new host organism.

The environment in which this transformation is to take place is highly competitive, thus even small fitness deficits would make it likely that the transformed organism will be unable to survive and be transmitted. Transfer and survival would, of course be more likely if recombination were to take place at a time when the bacteria were under antibiotic selection pressure, allowing rare and physiologically disadvantaged recombinants with an unfavourable adaptive advantage to survive because of a selective advantage to which the marker encodes resistance. The spectrum of the antibiotic resistance that is encoded by the marker and the degree of their use is important: in the case of *nptII*, neomycin and kanamycin are unlikely to be present in the environment where transformation takes place to provide a selection advantage. Thus, the adaptation deficit imposed by the transfer of an antibiotic-resistance marker would be likely to raise a substantial evolutionary barrier.

nptII in the context of aminoglycoside chemotherapy

The aminoglycoside class of antibiotics includes many natural or semisynthetic compounds.[84] They are multifunctional hydrophilic sugars that bind to the 30S ribosomal subunit, interfering with the elongation of the nascent protein chain by impairing the proofreading process, resulting in bacterial cell death.[85] The basis of kanamycin activity in plant cells is not clearly understood[86] but may be due to binding to prokaryote-like rRNA in the plastids.[87]

Aminoglycoside use has been declining over the past 20 years because less toxic alternatives with excellent antibactericidal activities are now available.[88] Neomycin

and kanamycin are now only used for skin, eye, and ear infections in human beings and animals, and pre-operative prophylaxis for digestive surgery in human beings. Aerosol administration of neomycin and kanamycin is largely confined to veterinary use. Streptomycin is still in use for the treatment of tuberculosis, but this has been declining because of the risk of injection in countries with a high incidence of tuberculosis and HIV coinfection.

Aminoglycoside resistance mechanisms

There are a number of aminoglycoside resistance mechanisms. First, single-step mutation to high-level resistance only occurs to streptomycin and generally is the result of a change in the *M tuberculosis* genes *rpsL* or *rrs*.[59,60] This event occurs at one mutation every 10^6 cell divisions but results in high-level resistance.[89,90] Second, uptake of aminoglycosides can be reduced by the presence of an active efflux pump—eg, Mex XY, which is of importance in resistance in *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis.[91] The third mechanism—the presence of aminoglycoside modifying enzymes, of which at least 11 genes families are known to cause resistance to kanamycin, neomycin, or paromomycin (table)—is the most important, because of its widespread nature and the degree of resistance encoded. Some aminoglycoside acetylases not included in the table are able to acetylate kanamycin, although not to the extent that they could provide resistance in the bacteria.[48] Finally, a novel mechanism thought to have been transferred to *Pseudomonas* spp from aminoglycoside-producing organisms has been described and mediates high-level resistance to multiple compounds.[92]

Overall resistance mediated by *nptII*, with its spectrum restricted to kanamycin and neomycin, appears to have little, if any, clinical significance.

Transfer of aminogylcoside resistance genes

Aminoglycoside resistance is readily transmitted between bacteria, especially in the hospital environment. Surveys of Enterobacteriaciae demonstrate that a wide range of aminoglycoside-modifying enzymes are carried and that almost all are located on transmissible DNA. For organisms that have gained a foothold in the intensive therapy unit and are causing hospital-acquired infections—eg, *A baumanii*, *Stenotrophomonas maltophilia*, and *P aeruginosa*—resistance to aminoglycosides is very common and in some strains universal. The epidemiology of the inexorable rise of such multidrug-resistant strains is dominated by intensive antibiotic use and breakdown in hygiene in the patient environment that favours the clonal spread of resistant organisms. Efforts to control the tide of resistance depend on encouraging appropriate prescribing and optimising hygiene measures. Resistance to streptomycin in *M tuberculosis* arises due to spontaneous mutation in chromosomal genes. Therefore, resistance mainly arises as a new event in patients who do not adhere to therapy and in a minority due to infection with previously resistant organisms.

Conclusion

Plants modified genetically have tremendous potential to improve human nutrition, but at what cost? The outcome of the introduction of antibiotic-resistance markers in GM plants depends on the gene flow in the resistance reservoirs connecting the fields where these plants grow and the bacteria that could be altered to resistance and be transferred to human beings. It is clear that fragments of DNA long enough to encode a resistance mechanism can survive in the field and in the animal gut. There is still no evidence of recombination events taking place in this environment, but this does not exclude the possibility that it does occur. However, it does suggest the flow of genes into the resistance reservoir from this source must be slow, since the number of new resistance events are dwarfed by those created by the human therapeutic environment. Should recombination events take place, they are likely to impose a physiological price that imposes a negative

Selective agent	Aminoglycoside modifying enzyme	Gene location	Cross resistance								
			Neomycin	Kanamycin	Paromomycin	Gentamicin	Amikacin	Tobramycin	Dibekacin	Netilmicin	Apramycin
Kanamycin	APH 3' I	Plasmid, transposon	R	R	R	..	..	..	..	..	..
	ANT 2''	Plasmid	..	R	..	R	..	R	R	..	..
	AAC(6')-Ie+ANT(2'')[57]	Plasmid	R	R	..	R	..	R	..	..	..
Neomycin	APH 3' II (*nptII*)	Transposon	R	R	R	..	..	..	..	..	..
	APH 3' III	Plasmid	R	R	R	..	A	..	..	..	..
	APH 3' IV	Chromosome	R	R	R	..	..	..	..	..	..
	APH 3' V	Chromosome	R	..	R	..	..	..	..	..	..
	APH 3' VI	Plasmid	R	R	R	..	R	..	..	..	..
	APH 3' VII	Plasmid	R	R	..	..	A	..	..	..	..
	ANT (4')-I	Plasmid, chromosome	..	R	..	..	R	R	..	..	..
Paromomycin	AAC 1	Plasmid	..	..	R	..	..	..	..	..	R

A=enzymatic activity not associated with clinical resistance; R=resistance

Table: **The major neomycin and kanamycin resistance genes in bacteria. Modified from reference 48**

Search strategy and selection criteria

Data for this review were identified by searches of PubMed and references from relevant articles, as well as the extensive personal files of the authors. Search terms included "bacterial fitness", "transgenic plants", "antibiotic resistance", and "gene flow". Searches using the names of prominent scientists in the field were also done, and the related articles facility of PubMed used. The selection of papers was not restricted by language.

selection coefficient on the recombinant organism. Only organisms that are also under selective pressure at the time of recombination are likely to survive, multiply, and overcome this barrier. By contrast with the size of the potential reservoir created by antibiotic-resistance marker mutational change, transmission of genetic elements encoding resistance is being facilitated by high antibiotic prescription rates. Thus, we conclude that antibiotic-resistance markers do not pose a substantial risk to human health because the contribution that recombinant bacteria might make—should the enormous barriers to transfer be overcome—is so small that its effect would be completely overwhelmed by the effect of resistance that arises through inappropriate prescribing in medical practice, transmission of mobile genetic elements between bacteria colonising patients, and hospital environments. Despite this conclusion, alternative selective systems should be favoured for future developments.

Conflicts of interest

PBG is retired but has in the past acted as a consultant for Monsanto. SHG has also acted as a consultant for Monsanto.

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