STATEMENT OF EFSA

EFSA-Q-2009-00589 and EFSA-Q-2009-00593

Consolidated presentation of the joint Scientific Opinion of the GMO and BIOHAZ Panels on the "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants" and the Scientific Opinion of the GMO Panel on "Consequences of the Opinion on the Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants on Previous EFSA Assessments of Individual GM Plants"[1]

Prepared by GMO and BIOHAZ Units

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Summary

The following summary provides a consolidated overview of the joint scientific opinion of the GMO and BIOHAZ Panels on the "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants" adopted on March 26, 2009 and the scientific opinion of the GMO Panel on "Consequences of the Opinion on the Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants on Previous EFSA Assessments of Individual GM Plants" adopted on March 25, 2009.

Following a request from the European Commission to the European Food Safety Authority (EFSA) the Panel on Genetically Modified Organisms (GMO) and the Panel on Biological Hazards (BIOHAZ) were asked to deliver a joint scientific opinion on the use of antibiotic resistance genes as marker genes in genetically modified (GM) plants. This opinion should take account of the previous opinion and the statement of the GMO Panel on the use of antibiotic resistance marker genes in GM plants intended or already authorised to be placed on the market and their possible uses as food and feed, for import and processing and for cultivation. It was asked whether the Opinion could explain the rationale leading to the conclusion of whether the use of each particular antibiotic resistance marker gene is likely or not to have adverse effects on human health and the environment and outline the reasoning

[1] On a request from European Commission, DG SANCO, Mandate No EFSA-M-2008-0411; issued on 14 May 2008.

leading to each conclusion. The opinion should also serve as a basis for the case-by-case safety assessment of each GM plant and its processed products.

The antibiotic resistance traits as present in GM plants and/or their derived products are evaluated on a case-by-case basis with respect to their safety for humans, animals and the environment by the GMO Panel according to the scientific principles expressed by the Directive 2001/18/EC of the European Parliament and the Council (EC, 2001) and detailed by the regularly updated guidance documents of EFSA (EFSA, 2006). The evaluation is based on molecular, biochemical, toxicological and environmental evidence.

The joint opinion of the GMO and BIOHAZ Panels (Annex 1) focuses on the two antibiotic resistance marker genes that are present in GM plants for which an application has been submitted to EFSA. One is functional in the plant (*aph(3')*-IIa = *npt*II, kanamycin/neomycin resistance); the other gene (*ant-(3'')*-Ia = *aad*A; streptomycin/spectinomycin resistance) is not expressed in the GM plants as the expression is regulated by a bacterial promoter not active in plants. The latter gene is used at the initial steps to develop the genetic constructs before introduction to the plant. An overview of relevant scientific literature is given and a qualitative risk assessment is provided. Whilst a detailed evaluation of *aph(3')*-IIa and *ant-(3'')*-Ia genes is included in the appendices, the opinion itself specifically addresses the indirect hazards.

From all the evidence gathered, the two Panels drew the following conclusions:

The transfer of antibiotic resistance marker genes from GM plants to bacteria has not been shown to occur either in natural conditions or in the laboratory in the absence of sequence identity in the recipient bacterial cell. Sequence identity is necessary to allow homologous recombination between the transformed DNA in the plant and bacterial DNA.

DNA transfer from GM plants to bacteria, if occurring, is considered to be of low frequency compared with gene transfer between bacteria.

Recent metagenomic analyses of total bacterial populations (including non-cultivable bacteria) have demonstrated that resistance determinants of kanamycin, neomycin and streptomycin are present in all environments investigated. Such resistance genes may be selected from this environmental reservoir and disseminated among bacteria.

The antibiotic resistance marker genes, *aph(3')*-IIa (*npt*II) and *ant(3'')*-Ia (*aad*A), in GM plants are of bacterial origin. These antibiotic resistance genes occur at different frequencies in different species, isolates and different environments, in naturally occurring bacteria. The spatio-temporal relationship between the prevalence of antibiotic resistance and selection pressure is not fully understood.

The presence of antibiotics and antibiotic usage in different environments are key factors in driving the selection and dissemination of antibiotic resistance genes.

Kanamycin and neomycin are both categorized by the WHO Expert Group on Critically Important Antimicrobials for Human Health as 'Highly Important Antimicrobial'. Kanamycin is used as a second-line drug for the treatment of infections with multiple drug-resistant tuberculosis (MTB). The increasing occurrence worldwide of "extensively drug-resistant" (XTB) isolates of MTB with resistance to second-line antibiotics such as kanamycin is a cause for global concern. The *npt*II gene has not been implicated in such resistance. The above WHO group has also categorised streptomycin as a 'Critically Important Antimicrobial', and spectinomycin as a 'Highly Important Antimicrobial'.

There are limitations related among others to sampling, detection, challenges in estimating exposure levels and the inability to assign transferable resistance genes to a defined source. The importance of taking these and other uncertainties described in this Opinion into account requires to be stressed.

Notwithstanding these uncertainties, the current state of knowledge indicates that adverse effects on human health and the environment resulting from the transfer of these two antibiotic resistance genes from GM plants to bacteria, associated with use of GM plants, are unlikely.

Two members of the BIOHAZ Panel expressed minority opinions on this last conclusion. Full details of the proposal for amendment of the mentioned conclusion are provided in Appendix D of Annex 1.

In addition, the European Commission requested EFSA to indicate the possible consequences of this new opinion on the previous EFSA assessments of individual GM plants containing antibiotic resistance marker genes. This aspect is addressed in the scientific opinion of the GMO Panel (Annex 2).

The GMO Panel has issued previously scientific opinions about the safety of two GM plant events that contain the *aph(3')*-IIa gene (*npt*II), i.e. maize MON 863 and hybrids and starch potato EH92-527-1. In the light of the new EFSA scientific opinion "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants", the GMO Panel is of the opinion that its previous assessments on GMOs containing this antibiotic resistance marker gene are in line with the risk assessment strategy described in the above opinion, and that no new scientific evidence has become available that would prompt the Panel to change its previous opinions.

Following adoption of these opinions by the respective Panels, EFSA consulted the Chairs of the GMO and BIOHAZ Panels as to whether the completion of the mandate would require a clarification of issues raised in the minority opinions of the joint scientific opinion (Letter addressed to the Chairs of the GMO and BIOHAZ Panels and to the Chair of the Joint Working Group - Annex 3). The Chairs responded by confirming that the scientific issues related to the minority opinions have already been extensively considered during the preparation of the joint scientific opinion and the formulation of the conclusions therein and thus, from a scientific perspective, further clarification of the joint scientific opinion is not required, nor is further scientific work needed at this time (Annex 4).

Key words: Directive 2001/18/EC, Regulation 1829/2003, GMOs, GM plants, antibiotics, antibiotic resistance marker genes, safety, food safety, human health, environment, horizontal gene transfer, *npt*II, *aad*A.

Background as provided by the European Commission

The Health & Consumer Protection Directorate-General (DG SANCO and DG ENV) of the European Commission gave a mandate to EFSA on 14 May 2008 for a 'consolidated opinion on use of antibiotic resistance marker genes used as marker genes in genetically modified plants'. The Commission letter annexed correspondence from Greenpeace (13 February 2008 and 13 September 2007) and from the Danish authorities (14 March 2008) related to the antibiotic resistance marker gene issue.

Terms of reference as provided by the European Commission

According to Article 29 of Regulation (EC) No 178/2002[2], EFSA was requested:

1. To prepare a consolidated scientific opinion taking into account the previous opinion and the statement on the use of antibiotic resistance marker genes in GM plants intended or already authorised to be placed on the market and their possible uses as food and feed, for import and processing and for cultivation.

This Opinion should explain the rationale leading to the conclusion of whether the use of each particular antibiotic resistance marker gene is likely or not to have adverse effects on human health and the environment and outline the reasoning leading to each conclusion. The opinion should also serve as a basis for the case-by-case safety assessment of each GM plant and its processed products.

2. To indicate the possible consequences of this new opinion on the previous EFSA assessments on individual GM plants containing antibiotic resistance marker genes.

EFSA was asked to work in close collaboration with the European Medicines Agency (EMEA) and any other appropriate scientific institutes having recognised international expertise in the field of antibiotic resistance in order to characterise the use and importance of the antibiotics for which these genes encode resistance.

Given the need to proceed in a timely manner with the outstanding applications/notifications of products containing antibiotic resistance marker (ARM) genes, the Commission initially set a deadline for the opinion to not later than 30 September 2008. From the request by EFSA, the deadline was extended until March 2009.

Approach taken to answer to the Terms of Reference

After having received this request from the European Commission, EFSA allocated the mandate to the GMO and BIOHAZ Panels. On March 26, 2009, The GMO Panel and the BIOHAZ Panel adopted jointly a Scientific Opinion entitled "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants", addressing Terms of Reference 1 (attached as Annex 1). Furthermore, on March 25, 2009, the GMO Panel adopted a Scientific Opinion entitled "Consequences of the Opinion on the Use of Antibiotic Resistance Genes as

[2] OJ L 31, 28.1.2002, p. 1.

Marker Genes in Genetically Modified Plants on Previous EFSA Assessments of Individual GM Plants" addressing Terms of Reference 2 (attached as Annex 2).

After adoption of these two opinions, EFSA consulted the Chairs of the GMO and BIOHAZ Panels whether the completion of the mandate would require a clarification on issues raised in the minority opinions of the joint scientific opinion (attached as Annex 3). The Chairs responded by confirming that the scientific issues related to the minority opinions have already been extensively considered during the preparation of the joint scientific opinion and the formulation of the conclusions therein and thus, from a scientific perspective, further clarification of the joint scientific opinion is not required, nor is further scientific work needed at this time (attached as Annex 4).

Both Scientific Opinions and the letter from the Chairs of the GMO and BIOHAZ Panels are presented in a consolidated form in this EFSA statement.

Assessment

The scientific assessment can be found under the Assessment sections in the Scientific Opinion entitled "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants" adopted jointly by the GMO Panel and the BIOHAZ Panel (Annex 1) and in the Scientific Opinion entitled "Consequences of the Opinion on the Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants on Previous EFSA Assessments of Individual GM Plants" adopted by the GMO Panel (Annex 2)

In the following sections, an overview of the conclusions of the two Scientific Opinions is presented.

Conclusions answering the Terms of Reference

ToR 1: *To prepare a consolidated scientific opinion taking into account the previous opinion and the statement on the use of antibiotic resistance marker genes in GM plants intended or already authorised to be placed on the market and their possible uses as food and feed, for import and processing and for cultivation.*

Conclusions from the Scientific Opinion entitled "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants" adopted jointly by the GMO Panel and the BIOHAZ Panel (Annex 1)

- The transfer of antibiotic resistance marker genes from GM plants to bacteria has not been shown to occur either in natural conditions or in the laboratory in the absence of sequence identity in the recipient bacterial cell. Sequence identity is necessary to allow homologous recombination between the transformed DNA in the plant and bacterial DNA.
- DNA transfer from GM plants to bacteria, if occurring, is considered to be of low frequency compared with gene transfer between bacteria.
- Recent metagenomic analyses of total bacterial populations (including non-cultivable bacteria) have demonstrated that resistance determinants of kanamycin, neomycin and streptomycin are present in all environments investigated. Such resistance genes may be selected from this environmental reservoir and disseminated among bacteria.
- The antibiotic resistance marker genes, *aph(3')*-IIa (*npt*II) and *ant(3'')*-Ia (*aad*A), in GM plants are of bacterial origin. These antibiotic resistance genes occur at different frequencies in different species, isolates and different environments, in naturally occurring bacteria. The spatio-temporal relationship between the prevalence of antibiotic resistance and selection pressure is not fully understood.
- The presence of antibiotics and antibiotic usage in different environments are key factors in driving the selection and dissemination of antibiotic resistance genes.
- Kanamycin and neomycin are both categorized by the WHO Expert Group on Critically Important Antimicrobials for Human Health as 'Highly Important Antimicrobial'. Kanamycin is used as a second-line drug for the treatment of infections with multiple drug-resistant tuberculosis (MTB). The increasing occurrence

worldwide of "extensively drug-resistant" (XTB) isolates of MTB with resistance to second-line antibiotics such as kanamycin is a cause for global concern. The *npt*II gene has not been implicated in such resistance. The above WHO group has also categorised streptomycin as a 'Critically Important Antimicrobial', and spectinomycin as a 'Highly Important Antimicrobial'.

- There are limitations related among others to sampling, detection, challenges in estimating exposure levels and the inability to assign transferable resistance genes to a defined source. The importance of taking these and other uncertainties described in this Opinion into account requires to be stressed.
- Notwithstanding these uncertainties, the current state of knowledge indicates that adverse effects on human health and the environment resulting from the transfer of these two antibiotic resistance genes from GM plants to bacteria, associated with use of GM plants, are unlikely.

Two members of the BIOHAZ Panel expressed minority opinions on the last conclusion above (See Appendix D of Annex 1).

ToR 2: *To indicate the possible consequences of this new opinion on the previous EFSA assessments on individual GM plants containing antibiotic resistance marker genes.*

Conclusions from the Scientific Opinion entitled "Consequences of the Opinion on the Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants on Previous EFSA Assessments of Individual GM Plants" adopted by the GMO Panel (Annex 2)

In the light of the new EFSA scientific opinion "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants", the GMO Panel is of the opinion that its previous assessments on GMOs containing this antibiotic resistance marker gene are in line with the risk assessment strategy described in the above-mentioned opinion, and that no new scientific evidence has become available that would prompt the Panel to change its previous opinions.

Documentation provided to EFSA

1. Letter from DG SANCO and DG ENV, dated 14 May 2008, concerning the mandate for the use of antibiotic resistance marker (ARM) genes used as marker genes in genetically modified plants (ref. SANCO/E1/SP/pm (2008) D/510274).
2. Enclosure 1. May 2008. Submitted by European Commission.

   Letter from Greenpeace to the Commissioner for Health and Food Safety, dated 13 February 2008, concerning the authorisation of GM BASF potato EH92-527-1 / Agriculture Council 18 February 2008.
3. Enclosure 2. May 2008. Submitted by European Commission.

   Letter from the Danish Minister for Food, Agriculture and Fisheries and Minister of Environment to Commissioner Vassiliou and Commissioner Dimas, dated 14 March 2008.
4. Enclosure 3. May 2008. Submitted by European Commission.

Email message from Greenpeace to Commissioner Dimas, dated 13 September 2007, concerning the Institute Pasteur study on antibiotic resistance and GM plants.

References

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EFSA, 2006. Guidance document of the Scientific Panel of Genetically Modified Organisms for the risk assessment of genetically modified plants and derived food and feed. European Food Safety Authority, ISBN: 92-9199-019-1, EFSA Journal 99, 1-94.

ANNEXES

Annex 1

European Food Safety Authority

The EFSA Journal (2009) 1034, 1-82

SCIENTIFIC OPINION

Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants[1]

Scientific Opinion of the Panel on Genetically Modified Organisms (GMO) and the Panel on Biological Hazards (BIOHAZ)

(Questions No EFSA-Q-2008-411 and EFSA-Q-2008-706)

Adopted on 11 March 2009 by the GMO Panel

and on 26 March 2009 by the BIOHAZ Panel

PANEL MEMBERS[*]

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[1] For citation purposes: Scientific Opinion of the Panel on Genetically Modified Organisms (GMO) and the Panel on Biological Hazards (BIOHAZ) on a request from the European Commission on the use of antibiotic resistance genes as marker genes in genetically modified plants. *The EFSA Journal* (2009) 1034, 1-81.

[*] (minority opinion) This opinion is not shared by 2 members of the BIOHAZ Panel, Dr. Christophe Nguyen-Thé, and Dr. Ivar Vågsholm. The objections raised concerned the text of the last conclusion of the document, also included in the summary section. In addition, Dr. Ivar Vågsholm proposed adding a new recommendation. Details of the proposal for amendment of the mentioned conclusion and the new recommendation, as well as the argumentation for the minority Opinions, are provided in Appendix D.

SUMMARY

Following a request from the European Commission to the European Food Safety Authority (EFSA) the Panel on Genetically Modified Organisms (GMO) and the Panel on Biological Hazards (BIOHAZ) were asked to deliver a scientific opinion on the use of antibiotic resistance genes as marker genes in genetically modified (GM) plants. The scientific opinion should take account of the previous opinion and the statement on the use of antibiotic resistance marker genes in GM plants intended or already authorised to be placed on the market and their possible uses as food and feed, for import and processing and for cultivation. It was asked whether the Opinion could explain the rationale leading to the conclusion of whether the use of each particular antibiotic resistance marker gene is likely or not to have adverse effects on human health and the environment and outline the reasoning leading to each conclusion. The opinion should also serve as a basis for the case-by-case safety assessment of each GM plant and its processed products.

The antibiotic resistance traits as present in GM plants and/or their derived products are evaluated case-by-case for their safety to humans, animals and to the environment by the GMO Panel according to the scientific principles expressed by the Directive 2001/18/EC of the European Parliament and the Council (EC, 2001) and detailed by the regularly updated guidance documents of EFSA (EFSA, 2006a). The evaluation is based on molecular, biochemical, toxicological and environmental evidence.

This opinion focuses on the two antibiotic resistance marker genes that are present in GM plants for which an application has been submitted to EFSA. One is functional in the plant (*aph(3')*-IIa = *npt*II, kanamycin/neomycin resistance); the other gene (*ant-(3'')*-Ia = *aad*A; streptomycin/spectinomycin resistance) is not expressed in the GM plants as the expression is regulated by a bacterial promoter not active in plants. The latter gene is used at the initial steps to develop the genetic constructs before introduction to the plant. An overview of relevant scientific literature is given and a qualitative risk assessment is provided. Whilst an detailed evaluation of *aph(3')*-IIa and *ant-(3'')*-Ia genes is included in the appendices, the Opinion itself specifically addresses the indirect hazards. The possible consequences of this new Opinion on previous EFSA assessments are addressed in a separate opinion by the GMO Panel, (http://www.efsa.europa.eu/EFSA/ScientificPanels/GMO/efsa_locale-1178620753812_GMOOpinions455.htm).

From all the evidence gathered, the Panels drew the following conclusions:

The transfer of antibiotic resistance marker genes from GM plants to bacteria has not been shown to occur either in natural conditions or in the laboratory in the absence of sequence identity in the recipient bacterial cell. Sequence identity is necessary to allow homologous recombination between the transformed DNA in the plant and bacterial DNA.

DNA transfer from GM plants to bacteria, if occurring, is considered to be of low frequency compared with gene transfer between bacteria.

Recent metagenomic analyses of total bacterial populations (including non-cultivable bacteria) have demonstrated that resistance determinants of kanamycin, neomycin and streptomycin are present in all environments investigated. Such resistance genes may be selected from this environmental reservoir and disseminated among bacteria.

The antibiotic resistance marker genes, aph(3')-IIa (*npt*II) and ant(3'')-Ia (*aad*A), in GM plants are of bacterial origin. These antibiotic resistance genes occur at different frequencies in different species, isolates and different environments, in naturally occurring bacteria. The

spatio-temporal relationship between the prevalence of antibiotic resistance and selection pressure is not fully understood.

The presence of antibiotics and antibiotic usage in different environments are key factors in driving the selection and dissemination of antibiotic resistance genes.

Kanamycin and neomycin are both categorized by the WHO Expert Group on Critically Important Antimicrobials for Human Health as 'Highly Important Antimicrobial'. Kanamycin is used as a second-line drug for the treatment of infections with multiple drug-resistant tuberculosis (MTB). The increasing occurrence worldwide of "extensively drug-resistant" (XTB) isolates of MTB with resistance to second-line antibiotics such as kanamycin is a cause for global concern. The *npt*II gene has not been implicated in such resistance. The above WHO group has also categorised streptomycin as a 'Critically Important Antimicrobial', and spectinomycin as a 'Highly Important Antimicrobial'.

There are limitations related among others to sampling, detection, challenges in estimating exposure levels and the inability to assign transferable resistance genes to a defined source. The importance of taking these and other uncertainties described in this Opinion into account requires to be stressed.

Notwithstanding these uncertainties, the current state of knowledge indicates that adverse effects on human health and the environment resulting from the transfer of these two antibiotic resistance genes from GM plants to bacteria, associated with use of GM plants, are unlikely.

Two members of the BIOHAZ Panel expressed minority opinions on this last conclusion. Full details of the proposal for amendment of the mentioned conclusion are provided in Appendix D.

Key words: Directive 2001/18/EC, Regulation 1829/2003, GMOs, GM plants, antibiotics, antibiotic resistance marker genes, safety, food safety, human health, environment, horizontal gene transfer, *npt*II, *aad*A.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The Health & Consumer Protection Directorate-General (DG SANCO and DG ENV) of the European Commission gave a mandate to EFSA on 14 May 2008 for a 'consolidated opinion on use of antibiotic resistance marker genes used as marker genes in genetically modified plants'. The Commission letter annexed correspondence from Greenpeace (13 February 2008 and 13 September 2007) and from the Danish authorities (14 March 2008) related to the antibiotic resistance marker gene issue.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

According to Article 29 of Regulation (EC) No 178/2002[2], EFSA was requested:

1. To prepare a consolidated scientific opinion taking into account the previous opinion and the statement on the use of antibiotic resistance marker genes in GM plants intended or already authorised to be placed on the market and their possible uses as food and feed, for import and processing and for cultivation.

This Opinion should explain the rationale leading to the conclusion of whether the use of each particular antibiotic resistance marker gene is likely or not to have adverse effects on human health and the environment and outline the reasoning leading to each conclusion. The opinion should also serve as a basis for the case-by-case safety assessment of each GM plant and its processed products.

2. To indicate the possible consequences of this new opinion on the previous EFSA assessments on individual GM plants containing antibiotic resistance marker genes.

EFSA was asked to work in close collaboration with the European Medicines Agency (EMEA) and any other appropriate scientific institutes having recognised international expertise in the field of antibiotic resistance in order to characterise the use and importance of the antibiotics for which these genes encode resistance.

Given the need to proceed in a timely manner with the outstanding applications/notifications of products containing antibiotic resistance marker (ARM) genes, the Commission initially set a deadline for the opinion to not later than 30 September 2008. From the request by EFSA, the deadline was extended until March 2009.

APPROACH TAKEN TO ANSWER TO THE TERMS OF REFERENCE

After receiving this request from the European Commission, EFSA allocated the mandate to the GMO and BIOHAZ Panels. The present Scientific Opinion addresses the first Term of Reference. The second Term of Reference, is addressed by a Scientific Opinion adopted by the GMO Panel (http://www.efsa.europa.eu/EFSA/ScientificPanels/GMO/efsa_locale-1178620753812_GMOOpinions455.htm).

[2] OJ L 31, 28.1.2002, p. 1.

ACKNOWLEDGEMENTS

The European Food Safety Authority wishes to thank the members of the Joint GMO-BIOHAZ Working Group for the preparation of this opinion: Vittorio Silano (Chair), John Daniel Collins, Lieve Herman, Sirpa Kärenlampi, Hilde Kruse, Harry Kuiper, Kaare Nielsen, Christoph Nguyen-Thé and John Threlfall. The European Medicines Agency (Bo Aronsson and Christian Friis), and the European Centre for Disease Prevention and Control (Dominique Monnet), Hans-Jörg Buhk, Boet Glandorf, John Heritage, Christoph Tebbe, and Jan Dirk van Elsas are also acknowledged for their contributions to the Opinion. EFSA also kindly acknowledges the exchange of views with Patrice Courvalin (Institut Pasteur).

ASSESSMENT

1. Introduction

During the process of genetic modification, as defined by Directive 2001/18/EC (EC, 2001), marker genes are commonly used to facilitate the selection of transformed cells among the vast majority of untransformed cells. Marker genes encoding proteins that confer antibiotic resistance were first applied in the genetic modification of bacteria for fermentation of commercial products (*e.g.* amino acids, enzymes, antibiotics). The use of these marker genes was later extended to the genetic modification of fungi, plants and to human gene therapy.

The underlying question to be addressed is to what extent, if any, cultivation of genetically modified (GM) plants in which bacterial antibiotic resistance genes have been introduced as selectable markers contributes to the development of antibiotic-resistant bacteria of clinical importance.

Treatment of human and animal as well as some plant bacterial infections has been compromised worldwide by the emergence of bacteria with resistance or decreased susceptibility to one or more antibiotics other than those to which the organisms are inherently resistant (Roberts and Simpson, 2008). A requirement for more prudent use of antibiotics to decrease the contribution of selection pressure towards increasing resistance has been strongly voiced (Taubes, 2008). The origin(s) of such resistance is not always known. In people and animals as well as plants there exist bacterial populations whose genetic composition and diversity allows the expression of such resistance as a direct response to selective pressures. This ability is also found in the environmental bacterial flora. Critical to this problem are transferable antibiotic resistance genes in bacteria already present in the environment and also in pathogenic bacteria.

There is an increasing occurrence of antibiotic resistance globally, both in human and in veterinary medicine. This has two consequences, namely: 1) that antibiotics now available for clinical use will become increasingly compromised, and 2) any classification of antibiotic drugs as critical will change to incorporate new scientific information on resistance and new antimicrobial agents (WHO, 2007). In these circumstances and taking into account the limitations of current knowledge, bacterial resistance to any antibiotic cannot be discounted. This Opinion addresses the dynamic context and inherent uncertainties of these aspects.

Actions taken by the EU to reduce antimicrobial resistance in the food production chain include the decision to remove from the market antibiotics used as growth promoters in food production animals after January 1st 2006 (EC, 2003). The subject of antimicrobial resistance in relation to food safety has also been addressed in various Scientific Opinions (EFSA, 2005a; updated in EFSA, 2008a, b). In these Opinions EFSA has emphasized the need of reducing the spread of the genetic determinants for antimicrobial resistance in the food chain. In the case of viable micro-organisms used as, or in, feed additives, EFSA considered that they "should not add to the pool of antimicrobial resistance genes already present in the gut bacterial population or otherwise increase the risk of transfer of drug resistance". Moreover in the EFSA Opinion 2005, updated in EFSA, 2008b, EFSA concluded that whenever such microorganisms possessed resistance to antimicrobials, the risk would be minimal if "the gene (or genes) conferring resistance is (are) not associated with mobile genetic elements".

The use of antibiotic resistance genes as marker genes in GM plants has been subject to two safety assessments by EFSA: the scientific opinion of April 2004 on the use of antibiotic resistance genes as marker genes in genetically-modified plants (EFSA, 2004a), and the scientific statement of March 2007 on the safe use of the *npt*II antibiotic resistance marker gene in genetically-modified plants (EFSA, 2007a). Considering the number of pending applications and renewals concerning GM plants containing an antibiotic resistance marker gene and the GM plants already placed on the market within the European Union (Appendix C), it is important to provide an up-to-date view on this issue.

The present opinion takes into account the previous opinions of the GMO Panel (EFSA, 2004a; 2007) and includes a risk assessment of the potential dissemination of antibiotic resistance marker genes from GM plants into bacteria based on current scientific knowledge. There is an increasing understanding of the origin, evolution and ecology of antibiotic resistance genes and this has been acknowledged in this Opinion.

In the genetic modification of plants, the marker genes are introduced to aid the selection of traits for which screening would otherwise be too laborious to be of practical value. Antibiotic resistance genes may also be adventitiously present, as remnants from the initial construction of the gene cassettes in a bacterium. During the genetic modification of plants, these marker genes may be stably integrated in the genome of the plant cells and are inherited like any other plant gene. Antibiotics are not applied to the plant after the initial selection step.

This opinion focuses on the two antibiotic resistance marker genes that are present in GM plants for which an application has been submitted to EFSA (Appendix C). One is functional in the plant (*aph(3')*-IIa = *npt*II, kanamycin/neomycin resistance, and originates from the Transposon Tn*5* which was originally isolated from *Klebsiella pneumoniae*, but was subsequently found in a number of bacteria including *Escherichia coli* [Appendix A]); the other gene (*ant-(3'')*-Ia = *aad*A; streptomycin/spectinomycin resistance, which originates from the plasmid R538-1 of *Escherichia coli* [Appendix B]), is used at the initial steps to develop the genetic constructs before introduction to the plant. The latter gene is not expressed in the GM plants as the expression is regulated by a bacterial promoter not active in plants.

Hazards associated with the use of bacterial antibiotic resistance genes in the generation of GM plants can be direct or indirect. The **direct** hazards include:

1) toxicity or allergenicity of protein encoded by the DNA;

2) toxicity or allergenicity of compounds arising from the activity of the novel protein;

3) possibility that clinical therapy of orally-administered antibiotics could be compromised through antibiotic-inactivating proteins present in food derived from a GM plant containing an antibiotic resistance marker gene.

The **indirect** hazard for humans and animals is the transfer of resistance from a plant to a bacterium pathogenic to humans or animals, either directly, or *via* a commensal bacterium.

The antibiotic resistance traits as present in whole GM plants are evaluated case-by-case for their safety to humans, animals and to the environment by the GMO Panel according to the scientific principles expressed by the Directive 2001/18/EC of the European Parliament and the Council (EC, 2001) and detailed by the regularly updated guidance documents of EFSA (EFSA, 2006a). The evaluation is based on molecular, biochemical, toxicological and environmental evidence.

Whilst an overall evaluation of *aph(3')*-IIa and *ant-(3'')*-Ia genes is included in the appendices, the main focus of this Opinion is on the indirect hazards. The present document is an update of the scientific progress.

There are indications that the use of antibiotic resistance marker genes in feed and food production may be superseded by newer technologies now under development.

2. Present state of scientific knowledge

2.1. Direct and indirect evidence for gene transfer from plant material to bacteria

Many studies have demonstrated the occurrence and significance of interspecies horizontal gene transfer (HGT) in bacterial adaptation and evolution (see 2.1.3.). Horizontal acquisitions of eukaryotic genes has rarely been observed in sequenced bacterial genomes (see 2.1.1.2.) although free-living bacteria are likely to be exposed to a variety of DNA sources present in their environment (Nielsen *et al*., 2007a). The absence of frequent horizontal transfer events from eukaryotic DNA sources into bacteria suggests the presence of discriminate mechanistic, functional and/or selective barriers.

It is generally accepted that the mechanism for gene flow from plants to bacteria would involve the capture of DNA released from GM plant material by competent bacteria *via* transformation. However, there are major barriers that restrict the likelihood of functional gene transfer (Keese, 2008). The most important barriers, identified through experimental exposures of bacteria to foreign DNA fragments, are the lack of efficient cellular uptake and integration mechanisms of non-homologous DNA fragments, the low likelihood of transfer of functional DNA, due to the presence of introns and different regulatory pathways in eukaryotes, and the predicted absence of positive host selection conferred by the acquired DNA (Bennett *et al*., 2004; Nielsen and Daffonchio, 2007; Pontiroli *et al*., 2007; Keese, 2008). These barriers limit the observable number of evolutionary successful events of eukaryotic DNA acquisitions in bacteria.

In this chapter, the main studies that provide direct and indirect evidences for the potential occurrence of gene transfer from plants to bacteria are reviewed and the methodological limitations and advantages of the approaches are discussed.

2.1.1. Approaches to investigate plant material-to-bacteria gene transfer

The only scientifically substantiated pathway for possible horizontal transfer of chromosomally-integrated antibiotic resistance marker genes from plants to bacteria is the direct uptake of DNA by naturally competent bacteria (in a process called natural transformation) (De Vries and Wackernagel, 1998; Gebhard and Smalla, 1998; Nielsen *et al*., 1998). Bacteria naturally encounter plant DNA as this is passively released from decaying or mechanically damaged plant tissues (Kay *et al*., 2002; Ceccherini *et al*., 2003; Tepfer *et al*.,

2003). It is well-known that saprophytic bacteria are among the main degraders of plant macromolecules including DNA (Nielsen *et al*., 1998, 2007a; Keese, 2008).

Experimental evidence for the likelihood of bacterial uptake of antibiotic resistance genes can be drawn from studies on DNA-exposed bacteria in the laboratory or in natural bacterial communities, e.g. in the gastrointestinal system or agricultural fields (see section 2.1.1.1). Table 1 lists several peer-reviewed studies on the uptake of plant DNA into the cytoplasm of competent bacteria. These studies indicate that bacteria lack mechanisms for effective integration of foreign linear DNA fragments and, hence, are unable to ensure its heritable stability in all cases. Only when some bacterial strains have been genetically modified to contain recombination-facilitating integration systems, can uptake of plant-derived DNA be shown experimentally in the laboratory. Such events have not been reported in bacterial communities in the field or gastrointestinal system.

Identification of evolutionary successful DNA transfers from plants to bacteria based on comparative genome analysis are of value to the assessment, as such analysis provides information about the extent to which eukaryotes and bacteria share genes on an evolutionary time scale and about the extent to which the eukaryotic genes contribute to bacterial genome composition and evolution (see section 2.1.1.2).

2.1.1.1. Experimental evidence

The key outcomes of studies in which the possible occurrence of horizontal gene transfer from plants to bacteria have been experimentally investigated are summarized below. A distinction is made between the studies depending on whether or not facilitated recombination (FR) systems (also called marker rescue systems) have been used to promote the integration of antibiotic resistance marker gene fragments into the bacterial genome (Table 1).

Studies on horizontal gene transfer into bacterial monocultures

The underlying assumption behind the early studies (conducted up to 1998) on the potential transfer of antibiotic resistance marker genes into competent bacteria was that such uptake can occur spontaneously, either by illegitimate recombination or through randomly occurring similarity between the plant DNA fragments carrying the antibiotic resistance marker genes and the genome of the recipient bacterium. As seen in Table 1, none of the studies conducted with bacterial recipients in the absence of introduced identity have been able to demonstrate experimentally the uptake and integration of antibiotic resistance marker genes into bacteria (Nielsen *et al*., 1997).

Table 1. Studies on the potential for horizontal acquisition of plant antibiotic resistance marker (ARM) genes by bacteria (monocultures or natural bacterial communities) (modified from Nielsen *et al.*, 2001). The key outcomes of investigations with homology-facilitated recombination systems are presented in the dark grey column, and those exposing naturally occurring bacteria in the light grey column.

Donor species	Marker gene	Recipient species/ environment	Experimental conditions	Integration of ARM genes into the recipient bacterium based on		Reference
				Homologous recombination events with inserted recombination sites in recipient	Random or illegitimate recombination events	
Solanum tuberosum (potato)	*amp*	*Erwinia chrysanthemi*	Plant material infected with Erwinia		No ($<10^{-9}$)	Schlüter *et al.*, 1995
Nicotiana tabacum (tobacco)	*acc*I	*Agrobacterium tumefaciens*	Plant galls transformed with Agrobacterium		No ($<10^{-11}$)	Broer *et al.*, 1996
Beta vulgaris (sugarbeet), *Solanum tuberosum* (potato)	*npt*II	*Acinetobacter baylyi*	In vitro exposure to plant DNA		No ($<10^{-11}$)	Nielsen *et al.*, 1997
Solanum tuberosum (potato), *Brassica napus* (rapeseed), *Lycopersicon esculentum* (tomato), *Beta vulgaris* (sugarbeet)	*npt*II	*Acinetobacter baylyi*	In vitro exposure to plant DNA	Yes[a] (10^{-8})	No	De Vries and Wackernagel, 1998
Beta vulgaris (sugarbeet)	*npt*II	*Acinetobacter baylyi*	In vitro exposure to plant DNA	Yes[a] (10^{-9})	No	Gebhard and Smalla, 1998
Beta vulgaris (sugarbeet)	*npt*II	Silt loam soil	Plants grown on field		No	Gebhard and Smalla, 1999
Nicotiana tabacum (tobacco)	*acc*I	Sandy clay-loam soil	Plants grown on field		No	Paget *et al.*, 1998
Beta vulgaris (sugarbeet)	*npt*II	*Acinetobacter baylyi*	Exposure to plant DNA in sterile soil microcosm	Yes[a] (10^{-8})	No	Nielsen *et al.*, 2000
Nicotiana tabacum (tobacco), *Solanum lycopersicum* (tomato)	*npt*II, *aad*A, *aac*1-IV	*Ralstonia solanacearum*	In vitro and in planta exposure to plant DNA	No ($<1.6 \times 10^{-9}$ in vitro; $<4.4 \times 10^{-9}$ in planta)		Bertolla *et al.*, 2000
Solanum tuberosum (potato)	*npt*II	*Acinetobacter baylyi* and *Pseudomonas stutzeri*	Purified DNA	Yes, up to 10^{-4}	No	de Vries *et al.*, 2001
Nicotiana tabacum (tobacco)	*aad*A, transplastomic	*Acinetobacter baylyi*	Purified DNA and crushed plant tissue	Yes, 10^{-8} to 10^{-6}	No ($<10^{-8}$)	Kay *et al*, 2002
Solanum tuberosum cv. Apriori (potato)	*npt*II	*Bacillus subtilis*	Purified tuber DNA	No		Kharazmi *et al.*, 2002

Nicotiana tabacum (tobacco)	*aad*A, trans-plastomic	*Acinetobacter baylyi*	Purified DNA	Yes, up to 10^{-4}		Ceccherini *et al*., 2003
Solanum tuberosum (potato)	*npt*II	*Acinetobacter baylyi*	Extracted DNA from soil exposed to plant	Yes, (approx. 10^{-8})	No	de Vries *et al*., 2003
Beta vulgaris (sugarbeet)	*npt*II	*Pseudomonas stutzeri*	Extracted DNA from soil exposed to plant	Yes,		Meier and Wackernagel, 2003
Six species of donor plants	*npt*II nuclear or transplastomic	*Acinetobacter baylyi*	Disrupted leaves and intact plant material	Yes, various freq. (approx. 10^{-8})	Not detected	Tepfer *et al*., 2003
Zea mays (maize)	*bla*	Soil bacteria	Plants grown on field		No	Badosa *et al*., 2004
Nicotiana tabacum (tobacco)	*aad*A, trans-plastomic	*Acinetobacter baylyi*	Purified DNA	Yes, 10^{-7}	No, $<10^{-10}$	de Vries *et al*., 2004
Solanum tuberosum (potato) and *Carica papaya* (papaya)	*npt*II	*Acinetobacter baylyi*	In vitro, purified DNA	Yes (approx. 10^{-8})		Iwaki and Arakawa, 2006
Nicotiana tabacum (tobacco)	*aad*A, trans-plastomic	*Acinetobacter baylyi*	In vitro exposure to plant DNA and crushed leaf material	Yes[a] (10^{-8})		Monier *et al*., 2007
Arabidopsis thaliana	*npt*II, GFP	*Acinetobacter baylyi*	In vitro with purified DNA, ground, chopped and whole leaves, sterile seedlings, ground roots, sterile and unsterile soil	Yes, 10^{-8} to 10^{-11}		Simpson *et al*., 2007b
Carica papaya (papaya)	*npt*II	*Acinetobacter baylyi*	DNA extracted from soil samples from papaya fields	No	No	Lo *et al*., 2007
Zea mays (maize)	*bla*	Soil bacteria	Plants grown in field for 10 years		No	Demaneche *et al*., 2008
Zea mays (maize)	*bla*	*Acinetobacter baylyi*	Purified DNA from fresh material, or extracted from silage or goat milk (feed maize)	Yes, 10^{-7} fresh material), no $<10^{-11}$ for DNA extracted from silage or milk		Rizzi *et al*., 2008a
Nicotiana tabacum (tobacco)	*aad*A, GFP, trans-plastomic	*Acinetobacter baylyi*	Defrosted leaf tissues with purified DNA of transplastomic tobacco	Yes, 10^{-9}		Rizzi *et al*., 2008b

a Values are usually given as the number of transformant bacteria obtained per total number of bacteria present in the experimental system used. However, not all cited studies provide transformation frequencies, nor are the frequencies adjusted to length of the transformation period. The numbers presented in the table should therefore be considered as rough estimates of approximate frequencies over a 24 h time period.

Schlüter *et al.* (1995) used the plant pathogenic bacterium *Erwinia chrysanthemi* as a recipient in experiments with transgenic potato material. The potato carried a complete copy of a bacterial plasmid including a bacterial marker gene (*uid*A). *Erwinia* lyses plant tissues with extracellular pectolytic enzymes and thus has an intimate association with the plant material. Even under optimised conditions, in which the marker gene was linked to a

functional origin of replication, evidence for plant to bacterium transfer was not found. The frequency for gene transfer was estimated to be below 2 x 10^{-17}, which is well below the limit of detection. No gene transfer from GM tobacco to *Agrobacterium tumefaciens* or from GM plants to *Ralstonia solanacearum* was accomplished either (Broer *et al*., 1996; Bertolla and Simonet, 1999). Uptake of plant-harboured bacterial DNA could not be detected in studies using unmodified *Acinetobacter baylyi* (De Vries *et al*., 2001; Nielsen *et al*., 1997, 2000) or in *Pseudomonas stutzeri* strains (De Vries *et al*., 2001).

Horizontal gene transfer of plant DNA to pure cultures of bacteria has been demonstrated in a few highly transformable bacterial species (*e.g.* the soil bacterium *Acinetobacter baylyi* or *Pseudomonas stutzeri)* under laboratory conditions when regions of homology to the plant-harboured bacterial marker gene were already present in the recipient bacterium. The first studies using these homology-facilitated integration systems were conducted by Gebhard and Smalla (1998) and De Vries and Wackernagel (1998). The process is dependent on homology between the plant transgene and the genetic material of the recipient bacterium. Transformation involves the correction of a partial deletion in the marker gene homologue in the bacterial genome by homologous recombination with a functional copy present in the plant genome. In both studies (Gebhard and Smalla, 1998; de Vries and Wackernagel, 1998), the plant DNA carried an *aph(3')*-IIa (*npt*II) gene encoding resistance to kanamycin and neomycin, and the recipient bacteria carried an inactivated homologue of the same gene controlled by a bacterial promoter. Transformants were recovered by kanamycin selection of transformed bacteria after plant DNA uptake and restoration of the bacterial *aph(3')*-IIa gene. When the DNA homology was removed, transformation frequency fell below the limit of detection. These results demonstrate that random homology between the antibiotic resistance marker gene fragment and the recipient bacterial genome was not present at a level that could facilitate recombination.

Several similar studies have also been based on homology-facilitated recombination systems. De Vries *et al.* (2001) exposed DNA from GM potatoes to *Acinetobacter baylyi* and *Pseudomonas stutzeri* and Nielsen *et al.* (2000) extended the findings using the marker rescue system to demonstrate DNA uptake in sterile soil. In a homology-based study of *Acinetobacter baylyi* cells containing a defective copy of the *aph(3')*-IIa gene (with 317 bp deleted), the bacteria were observed to incorporate DNA from GM plants (sugarbeet, tomato, potato, oilseed rape), leading to restoration of neomycin resistance (Nielsen *et al*., 2000). Simpson *et al*. (2007b) demonstrated transformation of *Acinetobacter baylyi* by purified plant DNA at rates of 5.5 x 10^{-11} transformants per recipient and also showed DNA uptake in non-sterile soil. In contrast, the addition of *aph(3')*-IIa-containing DNA from a transgenic plant to *Bacillus subtilis* did not lead to observable transformation events *in vitro* or *in situ* although a homology-based recombination system was present in the recipient bacteria (Kharazmi *et al*., 2002).

Kay *et al.* (2002) presented *in situ* studies on transplastomic plants that had recombinant DNA integrated in the chloroplast genome. In general, due to the relative differences in copy number, transfer frequency of antibiotic resistance marker genes from plants to bacteria, with facilitated recombination systems present, is expected to be approximately 1000 times higher when using DNA extracted from transplastomic plants compared to transgenic plants with genomic insertions. In all cases, no transfer has been observed in the absence of homology-facilitated recombination systems, the detection limit usually being between $<10^{-9}$ to $<10^{-13}$ transformants per total number of recipient cells.

Recently, Rizzi *et al.* (2008b) reported natural transformation of *Acinetobacter baylyi* on tobacco leaves defrosted and slightly abraded to stimulate natural plant decay in plant residuosphere. Purified DNA from transplastomic tobacco externally supplemented on defrosted leaf tissues gave 10^{-9} transformants per recipient cell in a homology-based recombination model. The transformed *A. baylyi* cells were localised *in situ* by microscopy to the interstices between epidermal cells and close to the stoma.

Advantages offered by the studies conducted with pure bacterial isolates are that the results represent direct evidence, the studies allow the identification and experimental testing of the presence of cellular barriers to the uptake of species-foreign DNA in bacteria, and the studies enable collection of experimental data showing that such uptake is not detectable with the currently used methods.

Limitations of the laboratory-confined studies of DNA uptake in pure bacterial isolates include: the low number (less than ten) of bacterial species examined; the frequent reliance on dense monocultures of bacteria; the use of artificial *in vitro*-based experimental systems that may not fully capture environmental conditions conducive to horizontal gene transfer; the lack of explicitly presented, testable and biologically-meaningful horizontal gene transfer hypotheses and, hence, inadequate choice of model systems and time perspectives (short DNA exposure times, most often less than 30 hours); unclear communication of the detection limits (Pettersen *et al*., 2005; Nielsen and Daffonchio, 2007). Some of these factors may over-estimate and some may under-estimate the likelihood of transfer.

In conclusion, horizontal gene transfer from GM plants into bacteria has only been observed when facilitated by the existence of DNA sequence homology between the transgene and the DNA of the recipient bacterium. Recovery of the plant DNA by naturally occurring bacteria has not been demonstrated, even when bacteria are exposed to DNA naturally released from plant tissues. The key barrier to stable uptake of antibiotic resistance marker genes from GM plants by bacteria is the extent of DNA sequence similarity.

Knowledge of the different steps in the transformation process influencing the likelihood of the process

Transformation includes the following steps: 1) release and persistence of the DNA from the donor; 2) uptake of the DNA by competent bacteria in the vicinity; 3) survival following exposure to destructive nucleases in the bacterial cell; 4) stable incorporation of the DNA in the recipient cell, and 5) expression of the incorporated DNA.

The literature concerning persistence of DNA in the environment was recently reviewed by Pontiroli *et al*. (2007). The persistence of extracellular DNA in the environment appears to be influenced by a number of biotic and abiotic factors, which favour DNA protection or which induce DNA degradation. Specific aspects are discussed below in relation to the different relevant environmental conditions.

To be transformed the DNA has to be taken up by competent bacterial cells. Some bacterial species become transformable in the natural course of their life cycle (*e.g. Streptococcus pneumonia*; Lunsford, 1998) while others (*e.g. Acinetobacter* sp. strain BD13, *Pseudomonas stutzeri, Neisseria gonorrhoeae*) are always in a competent state (reviewed by Lorenz and Wackernagel, 1994). Competence of bacterial species such as *E. coli* can be induced by chemical or physical conditions such as presence of $CaCl_2$, EDTA, temperature shifts, electric shocks *e.g*. by lightning (Davison, 1999; Demanèche *et al*., 2001; Cérémonie *et al.*, 2004, 2006). Natural competence was shown in *Escherichia coli* at low temperature and Ca^{2+}

concentrations, *i.e*. conditions which prevail in certain natural aquatic ecosystems (Baur *et al*., 1996). This competence was shown to be maintained for several weeks in resting cells. On the other hand, the potential dilution of DNA in these environments seems to constitute a barrier to the interaction with the recipient cells (Baur *et al.*, 1996). For *Azotobacter vinelandii* natural competence in soil was suggested to be induced under iron limitation, a condition that prevails in plant rhizosphere (Page and Grant, 1987). Studies on bacterial biofilms have demonstrated the role of cell signalling (quorum sensing) between bacterial aggregates in driving the development of competence and gene transfer. Several researchers suggest that competence would be enhanced in bacteria living in aggregates on the different surfaces of the plant (reviewed by Pontiroli *et al*., 2007). On the other hand, *in situ* soil conditions are not always conductive to metabolically-active bacteria, due to nutrient limitation. However, soil is heterogeneous and composed of a multitude of nutrient-rich microhabitats, which could foster competence development (Bertolla *et al*., 2000; Van Elsas *et al*., 2003; Pontiroli *et al*., 2007).

DNA is available in the environment mainly as double-stranded DNA. It is converted to single-stranded DNA during transport across the bacterial cell wall and cytoplasmic membrane. Once DNA enters the bacterial cell, it is generally assumed to be affected by exonucleases and restriction endonucleases that degrade incoming DNA. Degradation of incoming single-stranded foreign DNA by restriction endonucleases is questionable as restriction endonucleases generally recognize and cleave double-stranded DNA. In addition, it has been suggested that a leaky restriction barrier can lead to escape of the incoming DNA from restriction endonucleases (Nielsen *et al*., 1998, Pontiroli *et al*., 2007).

To be maintained in the bacterial cell, DNA must be capable of replication. Stabilisation of transformed DNA in bacteria can occur by forming an autonomously replicating element when an origin of replication would have been co-transferred to the GM plant and the replication functions are provided in the recipient cell, or by integration of the transformed sequences into the bacterial DNA. Absence of homologous sequences or origins of replication were identified as major barriers to horizontal gene transfer by transformation (Thomas and Nielsen, 2005; De Vries *et al*., 2001; Kay *et al*., 2002; Tepfer *et al*., 2003; Simpson *et al*., 2007a; Keese, 2008). Several studies report that a decline in the nucleotide sequence identity between donor and recipient leads to a significant decrease in the integration of donor DNA by homologous recombination (reviewed by Pontiroli *et al*., 2007, see Table 2).

DNA acquired by a bacterium is unlikely to be of significance unless it leads to phenotypic resistance. Phenotypic resistance relies on the expression of the antibiotic resistant protein and on the sensitivity of the antibiotic towards this protein. Expression of *aph*(3')-IIa will for example confer resistance to neomycin and kanamycin but not to other aminoglycosides of clinical use, like amikacin (Perlin and Lerner, 1986; Siregar *et al*., 1994). To be active the acquired DNA by the bacterium has to be expressed or has to alter the expression of endogenous genes. Bacterial gene expression depends on specific signals that are not universal between species, providing an additional molecular barrier (Keese, 2008). Expression signals can be provided either by the transformed DNA itself in the case that bacterial promoter sequences were co-transferred with the antibiotic resistance gene to the GM plant, or by a plant promoter that has promoter activity in bacteria. For example the P35S cauliflower mosaic virus promoter has been demonstrated to be active in *E. coli* (Assaad and Signer, 1990). Expression signals can also be provided by read-through from the signals already present in the recipient bacterial cell, or by genetic rearrangement often associated

with the presence of a transposable element (Davison, 1999). Uptake and recombination of acquired DNA fragments may also influence gene expression in bacteria. Examples may include the restoration of the antibiotic resistance gene in the bacterium, upregulation of expression of a gene encoding antibiotic resistance or alteration of the specificity of the antibiotic resistance (Nielsen *et al*., 1998). Besides the transcription initiation and termination signals, for a proper expression the bacterium has to be able to accomplish efficient translation and maintenance of functional protein product, correct folding and, when relevant, secondary modification of the protein product and appropriate interactions with other proteins and substrates. These all differ between organisms, and the extent of incompatibility often correlates with the degree of evolutionary distance. In general, the stringency of the barriers to horizontal gene transfer increases proportionally with genetic distance. Consequently, the frequency of horizontal gene transfer is much greater within species than between unrelated species (Fraser *et al*. 2007).

Plant production

DNA from plants will be released into the environment both during cultivation and after harvest as a result of lysis and senescence of plant material. Although most of the extracellular DNA is rapidly degraded, DNA has been found to persist in soil (Widmer *et al*., 1997; Bertolla *et al*., 2000; Ceccherini *et al*., 2003; De Vries *et al*., 2003; Zhu, 2006; Lo *et al*., 2007). The literature concerning persistence of DNA in the environment was recently reviewed by Pontiroli *et al*. (2007) and Nielsen *et al*. (2007b). The persistence of extracellular DNA in the environment appears to be influenced by a number of biotic and abiotic factors, which favour DNA protection or induce DNA degradation (Blum *et al*., 1997; Dale *et al*., 2002; Lo *et al*., 2007). DNA is altered and degraded by physical agents (heat, ultraviolet light), chemical factors (pH, reactive oxygen, heavy metals, *etc*.) and by enzymatic hydrolysis by plant or microbial nucleases.

Because DNA is chemically reactive due to its negative charge, it can form complexes with reactive environmental constituents such as minerals (Davison, 1999). Quartz sand, clay minerals, feldspar and heavy metals were shown to be binding substrates (Lorenz and Wackernagel, 1994). In addition, organic compounds, such as humic acids, were shown to complex DNA, and proteins and polysaccharides present in cellular debris may also protect DNA from enzymatic degradation (Paget and Simonet, 1994). Even if DNA is adsorbed tightly to minerals, it can still react with enzymes and transform bacteria (Paget and Simonet, 1994).

The persistence of DNA from GM plants in the field seems to vary from several weeks to several years (Widmer *et al*., 1997; Paget *et al*., 1998; Gebhard and Smalla, 1999; De Vries *et al*., 2003; Lo *et al*., 2007). A number of studies have been published in which the possible occurrence of bacterial transformants carrying plant-derived antibiotic resistance marker genes from fields planted with GM plants were screened (Paget *et al*., 1998; Gebhard and Smalla, 1999; Badosa *et al*., 2004; Demaneche *et al*., 2008).

Paget *et al*. (1998) performed a field trial to examine possible transfer of the *aac*C1 marker gene encoding beta-lactamase, from GM tobacco plants to indigenous soil bacteria. No transfer was detected among the 600 antibiotic-resistant bacteria isolated from the soil. Similarly, Gebhard and Smalla (1999) examined >4000 kanamycin-resistant bacterial soil isolates from a field trial with GM sugarbeet and found no evidence for horizontal transfer of the *npt*II gene from the plants. Direct analyses of total DNA from soil indicated that the

observed bacterial resistance was due to causes other than the *npt*II gene-carrying sugarbeet plants. Badosa *et al*. (2004) studied the putative bacterial acquisition of the *bla* gene (encoding a β-lactamase) from Bt176 maize in commercial fields in Spain during four years of cultivation in three climatic regions. No significant differences were found in total or ampicillin-resistant bacterial population levels between GM and non-GM fields. Of the 864 bacterial isolates analysed, none contained the ampicillin resistance gene that would have been acquired from Bt176 maize. The estimated total number of bacteria analysed was 10^8, corresponding roughly to the amount found in 1 gram of soil.

In a recent study, the prevalence and diversity of beta-lactamase genes in soil bacteria was analysed to estimate the potential impact that 10 years of cultivation of Bt176 maize harbouring the *bla* gene might have on the prevalence of *bla* genes in the soil and the emergence of new resistant bacterial strains (Demanèche *et al*., 2008). The total number of cultivable bacteria in the different test fields varied from 1.7 x 10^4 to 2.5 x 10^5 per gram of soil, of which ampicillin-resistant bacteria constituted 0.4 - 6.5% in the GM fields, 5.5 - 8.0% in the conventional maize fields, and as much as 54.4 - 69.6% in the prairie which was not disturbed by agricultural practices. The 576 ampicillin-resistant bacterial isolates collected from the soil exhibited a broad-spectrum β-lactam resistance. The *bla* gene fragments identified in several soil isolates were identical to the one in Bt176 maize, but those isolates were recovered in all three types of fields (GM, conventional and prairie). Metagenomic analysis (including the non-cultivable bacteria) of isolated DNA from soil, revealed ten DNA inserts that exhibited *bla* sequence identical to the one in Bt176 maize; five from GM field and five from prairie. The result suggested a natural prevalence of *bla* genes in the environment. The prevalence and resistance patterns of the resistant bacteria did not differ significantly between the GM and non-GM fields but rather depended on field location, sampling stage and year of study. The results thus indicated that some soil bacteria are naturally resistant to broad-spectrum β-lactam antibiotics, and that growing GM plants for 10 successive years on the same field has no measurable effect on the levels of penicillin resistant bacteria or their resistance spectrum. The high prevalence of bla_{TEM} alleles in soil communicated in this study conducted in France is in contrast to the lower levels observed in the study conducted in Spain (Badosa *et al*., 2004).

In a free-air, large-volume lysimeter experiment under natural conditions and with increasing selection pressure, no stable transformation was demonstrated of the glyphosate-tolerance *epsps* gene (encodes 5-enolpyruvylshikimate-3-phosphate synthase) from Roundup Ready soybeans to symbiotic, nodule-forming *Bradyrhizobium japonicum* (Wagner *et al*., 2008). In this 2-year study conditions comparable to normal farming were used.

Recently, bacterial soil populations in fields with GM-rice and non-GM-plants were compared in a controlled field plot study (Kim *et al*., 2008b). Glufosinate-resistant GM rice plants were grown, containing the phosphinothricin acetyltransferase gene (*bar* gene) originally derived from *Streptomyces hygroscopicus*. No *bar* gene sequence was detected in DNAs extracted from both cultured and uncultured soil bacterial fractions.

Food and feed chain

GM plant material intended for food and feed use is often subject to a variety of processing and storage regimes. These range from simple heat treatment (*e.g.* canning, drying) to the extraction of food ingredients. Studies on the susceptibility of DNA to processing and extraction regimes in fruit juice (Weiss *et al*., 2007), potato (Bauer *et al*., 2004; Kharazmi *et*

al., 2003), soymilk and maize (Kharazmi *et al*., 2003), and in a variety of food products have been published (reviewed by Klein *et al.*, 1998 and Van den Eede *et al*., 2004). Food processing and extraction of ingredients physically and chemically damages and degrades DNA, and this limits gene transfer (Kharazmi *et al*., 2003). For example, processing of plant material in temperatures above 95^0C for more than a few minutes has been shown to fragment DNA to the extent that genetic information is unlikely to be retained (Chiter *et al*., 2000).

Stability during processing is an inverse function of the length of DNA (Chen *et al*., 2005; Kharazmi *et al*., 2003). Naked DNA has been shown to be protected by meat matrix in sausages (Straub *et al*., 1999), by maltol, octyl gallate and spermidine in potato (Bauer *et al*., 2004) and by individual food components such as arginine and biogenic amines (Van den Eede *et al*., 2004). Only in a few studies has the potential for natural transformation been examined in food products. The development of competence and natural transformation of *Bacillus subtilis* has been reported in milk with added bacterial chromosomal DNA (Zenz *et al*., 1998; Kharazmi *et al*., 2002). We are not aware of any studies in which the potential transfer of antibiotic resistance marker genes from plants to bacteria in the food or feed chain has been experimentally investigated.

Gastrointestinal system

One hypothetical route for the transfer of antibiotic resistance marker genes of bacterial or other origins from plant material to bacteria is by the transfer of the DNA of such genes, consumed in food or feed, to the high number of bacteria present in the gastrointestinal tract. These exposure scenarios include both vertebrates and invertebrates that feed on plants or processed plants and plant ingredients above or below ground, pollinators, and humans (Gay and Gillespie, 2005; Keese, 2008).

A key prerequisite for plant DNA to be a substrate for competent bacteria in the gastrointestinal tract is the physical stability of minimum gene-sized DNA fragments in relevant locations. The persistence in the digestive tract (from mouth to colon) of DNA in food depends on enzymatic degradation and on chemical degradation by low pH conditions. The mouth and oesophagus are likely to have the highest concentrations of intact DNA entering via the diet. Free bacterial DNA has been shown *in vitro* to survive for ten minutes (between 35 and 61 % had been degraded) in human saliva (Mercer *et al*., 1999). Duggan *et al.* (2000) performed *in vitro* experiments with sheep saliva and rumen fluid, concluding that DNA remained available for transformation in the oral cavity but was rapidly inactivated further down the gastrointestinal tract. There are studies that demonstrate that plasmid DNA may persist in the gastrointestinal tract and may be biologically active (Wilcks *et al*., 2004). Under normal physiological conditions, *in vitro* and *in vivo* experiments have led to the assumption that naked DNA would not fully survive the stomach (Ferrini *et al*., 2007) on passage through the lower gastrointestinal tract (Beever and Kemp, 2000; Alexander *et al*., 2004). In the stomach, DNA would be chemically degraded by the low pH (exceptions are individuals with achlorhydria or hypochlorhydria in which the production of gastric acid is absent or low, respectively), while in the lower intestine pancreatic nucleases and other enzymatic activities from animal and microbial origin would play a role [reviewed by Jonas *et al*. (2001) and Van den Eede *et al*. (2004)].

In contrast to naked DNA, plant-derived DNA would be somewhat protected from degradation. 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 more slowly than pure DNA in human intestinal simulation, which was attributed to protection by the food matrix. In *in vivo* experiments with broilers fed Bt maize the *Cry1A(b*) gene was degraded to fragments smaller than 500 bp (Rossi *et al*., 2005). Similarly, Chambers *et al.* (2002) fed chickens with GM maize to explore the *in vivo* fate of the bacterial ampicillin resistance gene bla_{TEM} in bacteria and GM maize. The gene was found in the stomach contents but not in the lower intestine of animals fed GM maize.

Research on bacterial competence and transformation has been performed with culturable model bacteria. Several bacteria found in the human gut can develop competence when grown in the laboratory. However, no studies have been able to show that bacteria residing naturally in the gastrointestinal tract of higher animals develop competence *in situ* (Nordgård *et al*., 2007). Little attention has been given to the anaerobic fraction. Also the non-culturable bacteria, which make up 90% or more of the population in the gastrointestinal tract and in the soil, are challenging to examine experimentally for the occurrence of rare horizontal gene transfer processes (Strätz *et al*., 1996). In the gastrointestinal tract, inhibitory effects of rumen fluid and ovine saliva on the development of competence by *Streptococcus bovis* (Mercer *et al*., 1999) and of colon contents on the natural transformation of *Acinetobacter baylyi* (Nordgård *et al*., 2007) have been described.

Netherwood *et al.* (2004) reported a low-frequency transfer of a small fragment (180 bp) of an introduced gene (*epsps* conferring herbicide tolerance) derived from GM soybean to microorganisms within the small intestine of human ileostomists (individuals in whom the terminal ileum is resected and digested material is diverted from the body to an ileostomy bag). One to three copies of the small fragment per 10^6 bacteria were detected in samples taken from three of the seven ileostomics. In a trial using volunteers with an intact gastrointestinal tract no transgenic DNA was detected in the faeces following the consumption of a meal containing GM soya. A complicating factor in the study was that the *epsps* fragment had transferred into some gut bacteria prior to the start of the study.

The possibility of horizontal transfer of the *epsps* gene to ruminal bacteria was studied *in vitro* in ruminal fermentation with GM (Roundup Ready) canola meal (Reuter *et al*., 2007). It was shown that high concentrations of glyphosate may inhibit fermentation activity and, consequently, may exert selective pressure on rumen bacteria. Because bacteria may also possess EPSPS activity, exposure to glyphosate may favour those bacteria that possess or acquire the *cp4 epsps* transgene and thereby overcome glyphosate-mediated inhibition of biosynthesis of aromatic amino acids. Extensive polymerase chain reaction (PCR) assays provided no evidence of acquisition of *epsps* by feed- or fluid- associated bacteria during fermentation, even in the presence of a high concentration of glyphosate, which is higher than that occurring in the rumen *in vivo*.

Nordgård *et al*. (2007) did not observe any transformation of the naturally competent *Acinetobacter baylyi* bacterium present in various gut locations (in colonised germ-free mice) after introducing bacterial DNA containing the *aph(3')*-IIa gene in the feed. Transformants could not be detected even after a week of weak positive selection with kanamycin added to the drinking water. It should be noted that the total bacterial population numbers were low, thereby limiting the likelihood of detecting rare horizontal gene transfer events in single mice models.

Conclusion of studies of horizontal gene transfer (HGT) into bacterial communities

Advantages offered by the studies of the potential for DNA uptake in natural bacterial communities are that the results obtained represent direct evidence.

Limitations in screening DNA uptake in natural bacterial communities (*e.g.* in soil or in the gut) include:

- the low overall number of bacterial communities and studies conducted, the lack of explicitly presented and testable horizontal gene transfer hypotheses, resulting in inadequate experimental sampling design;
- limited ability to prove uptake of DNA in the unculturable fraction, limited focus on anaerobic bacteria, a highly limited coverage of locations and time points, and the limited attention given to selection in driving the population dynamics of rare transformants (Nielsen and Townsend, 2004; Heinemann and Traavik, 2004); and
- the limits in the sensitivity of the methods used to detect such transfer in natural ecosystems, considering the extent of exposure of the natural bacterial population to antibiotic-resistance marker genes introduced by the cultivation of GM plants when introduced on a global scale.

To summarise, transfer of antibiotic resistance marker genes from GM plant material to bacteria has not been demonstrated. The limited efficiencies of the investigation protocols used in the field studies conducted in an environmental context and the inability to assign the resistance to a defined source are limiting factors when drawing conclusions.

2.1.1.2. Evidence from genomic databases for horizontal gene transfer from plants to bacteria

In evolutionary terms, bacterial genomes are considered fluent and are partially re-assembled by acquiring foreign genes and deleting others (Ochman *et al*., 2000). Exchange of genes between bacteria by horizontal gene transfer is known to have played a crucial role in the evolution of bacteria and their genomes.

While many studies support the evolutionary significance of horizontal gene transfer between bacteria, eukaryotic genes in prokaryotic genomes are a rarity (Keeling and Palmer, 2008). There is no definitive report of DNA transfer from eukaryotes to bacteria[3]. Data from bioinformatics-based studies are of value for the assessment of transfer potential as they can provide information contributing to the understanding of the extent to which eukaryotes and bacteria share genes (on an evolutionary time scale), and to what extent their gene pool remains genetically and functionally separated.

[3] At the date of 24 September 2008 the public genome databases included more than 750 completed prokaryotic genomes (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). In the first annotation of the putative genes there are frequent cases where closest matches are found with eukaryotic genes, but these preliminary results have not manifested into demonstrations of horizontal gene transfer from eukaryotes to prokaryotes, as judged by the scientific publications interpreting the genomic sequencing data. For one functional gene, *i.e*., the phosphoglucose isomerase gene (*Pgi*), phylogenetic analyses indicated that the gene might have been transferred from a eukaryote to bacteria (Katz, 1996). The author calculated on the basis of molecular clock hypothesis that this transfer happened approximately 500 million years ago. The problem of lacking functionality of eukaryotic genes in bacteria was explained by the occurrence of *Pgi* sequences that lack introns found in some insects.

2.1.1.3. Recombination potential of antibiotic resistance marker genes from plants into bacterial genomes

To be maintained in the bacterial cell, DNA must be capable of replication. Stabilisation of plant DNA in bacteria can occur in two ways: (a) by forming an autonomously replicating element when an origin of replication has been co-transferred to the GM plant and the replication functions are provided by the recipient bacterium, or (b) by integration of plant sequences into the bacterial DNA by homologous, site-specific or non-homologous recombination (Nielsen *et al*., 1998; 2001).

The mechanisms for integration of foreign DNA into the bacterial genome are reviewed by Keese *et al.* (2008). The probability of integration of a plant-harboured antibiotic resistance gene into the genome of a microorganism depends, based on available evidence, almost exclusively on the degree of identity of the foreign DNA with that of recipient bacterium (Table 2). Absence of identical sequences or origins of replication were identified as major barriers to horizontal gene transfer by transformation (De Vries *et al*., 2001; Kay *et al*., 2002; Tepfer *et al*., 2003; Thomas and Nielsen, 2005; Simpson *et al*., 2007a; Keese, 2008). Several studies report that with decreasing sequence identity between donor and recipient there is a log-linear decline in the integration of donor DNA by homologous recombination (Pontiroli *et al*., 2007).

The gene context (i.e. the surrounding/neighbouring sequences) of the antibiotic resistance marker gene in the plant may thus influence the likelihood of gene transfer into bacterium and its expression in the bacterial cell. The presence of the following elements in the plant insert can be considered relevant: 1) bacterial origin of replication (broad vs. limited host range), 2) short sequences recognised by transposases and integrases, 3) bacterial vector sequences (*e.g.* derivatives of plasmid pBR322) bearing similarity to their ubiquitous natural bacterial progenitors (Bensasson *et al.*, 2004), and 4) bacterial expression signals upstream of the coding sequence of the antibiotic resistance marker gene. It should be noted that bacterial transposases or integrases do not recognize plant transposon sequences and transposition of a plant transposon or transposon sequence plus transposase in bacteria has not been shown. The presence of a plasmid origin of replication could only influence the stabilisation of the transformed DNA if 1) the linear DNA could circularise in the recipient bacteria, and 2) the replication functions are already present in the recipient bacterium (Kornackit *et al*., 1993; Bingle and Thomas, 2001).

It has been suggested that the likelihood of recombination of transgenes inserted into or flanked by chloroplast DNA in the GM plant could increase by the presence in bacteria of sequences homologous to chloroplast DNA (Nielsen *et al*., 2001; Kay *et al*., 2002; Monier *et al*., 2007). Although plant organellar genomes have a prokaryotic origin, their sequence similarity to present bacterial genomes is very low. Of 288 isolates of soil bacteria, 16 showed DNA sequence homology with the chloroplast genes *rbcL* and *accD*, although the weaker hybridisation signal intensity in comparison with the positive controls indicates that similarity was only partial (Monier *et al*., 2007).

Non-homologous or illegitimate recombination is known to be an inefficient process. Homology-facilitated illegitimate recombination in which the foreign DNA is linked on one side to a piece of DNA (150-200 bp) homologous to the recipient genome could facilitate integration of foreign DNA as determined for *Acinetobacter* (Table 2, De Vries and Wackernagel, 2002) and for *Streptococcus pneumoniae* (Prudhomme *et al*., 2002). The frequencies of transformation *in vitro* were up to six orders of magnitude lower than those

determined by transforming these strains with entirely homologous donor DNA (Pontiroli *et al*., 2007).

A recent study has shown that double illegitimate recombination can also occur in *Acinetobacter baylyi* in the absence of any homology (Table 2; Hülter and Wackernagel, 2008).

Table 2: Frequency of transformation and stabilisation of DNA in *Acinetobacter baylyi*

Length of donor DNA (bp)	Sequence identity (bp)[1]	Transformation frequency	Relative frequency	Reference[2]
5592	509(L)-440 (R) (2xHR)	$3.0 (\pm 0.4) \times 10^{-3}$	1	(1)
	(2xIR)	7.3×10^{-13}	2.4×10^{-10}	(1)
	704(I) (2xIR)	$3.0 (\pm 0.8) \times 10^{-10}$	1.0×10^{-7}	(1)
	774(R) (1xHR + 1xIR)	$1.0 (\pm 0.3) \times 10^{-5}$	0.003	(1)
	889(L) (1xHR + 1xIR)	$2.6 (\pm 0.3) \times 10^{-6}$	0.001	(1)
3720	915(L)-945(R) (2xHR)	$0.92 (\pm 0.1) \times 10^{-4}$	1	(2)
	1096(L) (1xHR+1xIR)	$1.1 (\pm 0.03) \times 10^{-8}$	1.2×10^{-4}	(2)
	(2xIR)	$\leq 1.3 \times 10^{-13}$	$\leq 1.4 \times 10^{-9}$	(2)
	311(L) (1xHR+1xIR)		8×10^{-6}	(2)
	183(L) (1xHR+1xIR)		4×10^{-7}	(2)
	99(L) (1xHR+1xIR)	$\leq 1.7 \times 10^{-11}$	$\leq 1.8 \times 10^{-7}$	(2)
1350	1149(L)-1425(R) (2xHR)	$4.0 (\pm 1.8) \times 10^{-3}$	1	(3)
	140(L)-298(R) (2xHR)	$3.5 (\pm 1.4) \times 10^{-5}$	8.8×10^{-3}	(3)
	147(L)-135(R) (2xHR)	$1.4 (\pm 1.7) \times 10^{-6}$	3.5×10^{-4}	(3)
	22(L)-88(R) (2xHR)	$1.1 (\pm 2.7) \times 10^{-7}$	2.8×10^{-5}	(3)
	39(L)-16(R) (2xHR)	$5.2 (\pm 8.3) \times 10^{-8}$	1.3×10^{-5}	(3)
2350	1149(L)-1425(R) (2xHR)		1	(3)
	140(L)-298(R) (2xHR)		1.8×10^{-4}	(3)
	147(L)-135(R) (2xHR)		7.0×10^{-6}	(3)
	22(L)-88(R) (2xHR)		5.6×10^{-7}	(3)
	39(L)-16(R) (2xHR)		2.6×10^{-7}	(3)

The donor DNA was linearised plasmid DNA. Hülter and Wackernagel (2008) and de Vries and Wackernagel, (2002) used *npt*II marker rescue system; Monier et al. (2007) used a system based on the rescue of the *aad*A gene.

[1]The sequence identity is given at the left (L), right or internal (I) of both sides of the deletion which have to be restored by recombination; HR: homologous recombination; IR: illegitimate recombination

[2] (1): Hülter and Wackernagel (2008); (2) de Vries and Wackernagel (2002); (3) Monier *et al*. (2007); values were kindly provided by the authors.

For high frequent transformation of purified plasmid DNA to *Acinetobacter baylyi* identity of about 1000 bp (about equally divided at both sides of the concerned gene) is necessary, resulting in two efficient homologous recombination events (Table 2). Dropping this identity to about 300 bp and 100 bp at both sides of the concerned sequence results in about 10^4 and 10^7 reduction in transformation frequencies, respectively. Identity at only one side of the concerned sequence, with a consequence of only one high-frequency homologous recombination event and one illegitimate recombination event, drops the frequency

substantially. Without any identity the transformation is possible by double illegitimate recombination, resulting in a drop of the frequency of 2.4 x 10^{10} times or below the detection limit of the bacterial experiment. The low transformation frequency (7 x 10^{-13}) measured for the process of double illegitimate recombination is well below the detection limits for all the experimental studies that have reported the potential horizontal transfer of antibiotic resistance marker genes from plants to bacteria.

Transformation with purified plant DNA in comparison with plasmid DNA decreases the transformation frequency 100 times (Monier *et al*., 2007). Transformation with crushed leaves decreased the frequencies at least 10^5 times.

In conclusion, available data indicate that integration of genes from plants into bacteria in the absence of DNA sequence identity is, at most, a rare event. Illegitimate recombination of GM plant-harboured antibiotic resistance genes into bacteria has not been recorded. On a theoretical basis, it can be anticipated that the bacterial origin of the marker genes and the genetic material immediately surrounding the antibiotic resistance marker gene in the plant may influence the stabilization of transformed DNA in bacteria.

2.1.1.4. Relevance of the data for the risk assessment

The relevance of studies drawn on for this opinion is high, as all the experimental investigations have directly examined the likelihood of horizontal gene transfer occurring from GM plants to exposed bacteria. Similarly, the outcomes of the field and gut-based screening studies are also relevant. The bioinformatics-enabled observations that horizontal gene transfer rarely occurs from eukaryotes (including plants) to bacteria can also be used to establish that such events are rare in an evolutionary context. The biological relevance of the exact frequencies observed in experimental studies is limited. This is because they have been recorded with various laboratory models and experimental conditions with unclear representativeness of conditions as they occur in nature. Moreover, the grading of frequencies as low or high should be understood as relative based on experimental limitations in the laboratory (Pettersen *et al*., 2005). Thus, no clear link has been established between gene transfer frequencies as measured in the laboratory and their biological impact in short- or long-term. The population dynamics of the descendants of the primary transformants cause the eventual impact. It is, therefore, necessary to understand how selection will shape the trajectories and population dynamics of the transformant cells for accurate impact predictions (Nielsen and Townsend, 2001; Pettersen *et al*., 2005).

The absence of identifiable horizontal transfer events in sequenced bacterial genomes from eukaryotic DNA sources into bacteria suggests the presence of mechanistic, functional and selective barriers. Concerning the transfer of antibiotic resistance marker genes from GM plants to bacteria, the lack of efficient integration mechanisms of non-homologous DNA fragments has been identified as the most important barrier in experimental studies. Uptake of plant-derived DNA could be shown experimentally only when bacteria have been genetically modified to contain acquired recombination-facilitating integration systems. As a consequence, the gene context of the antibiotic resistance marker genes in plants (presence of genetic elements favouring DNA stabilisation and expression) could theoretically influence the likelihood of gene transfer to bacteria and subsequent expression of antibiotic resistance in the bacterial cells. In the absence of recombination-facilitating integration systems, no

transfer of a DNA fragment with a length corresponding to a functional coding sequence from GM plants to bacteria has been observed.

In conclusion, current scientific evidence indicates that source-independent uptake of free DNA molecules into the cytoplasm of naturally-occurring bacteria can occur. Where efficient mechanisms for integration into bacterial genome are absent, stabilization of acquired traits is not detected.

Current scientific evidence indicates that the transfer from GM plants into bacteria and the stable integration, in bacteria, of antibiotic resistance marker genes derived from GM plants either does not occur or, if it has occurred, it has been below the limit of detection in all the experiments performed in the different ecosystems involved in the process. This conclusion is also supported by inference from bioinformatics studies.

The above conclusions are subject to the results and interpretation of the horizontal gene transfer studies as provided by a limited number of experimental investigations, the detection limits as defined by the experimental design, and the small sample sizes analyzed.

2.1.2. Bacterium-to-bacterium transfer of antibiotic resistance genes

2.1.2.1. Transfer mechanisms

In broad terms pathogenic bacteria become resistant to antibiotics other than those to which the organism is already inherently resistant by two different mechanisms – by spontaneous mutational events, or by gene acquisition.

The emergence of mutation-based resistance is often associated with extensive use of antibiotics in both human and veterinary medicine. Mutations (base changes, DNA deletions or insertions and inversions) alter the endogenous genes but do not add new genes to the genome (Courvalin, 2008). Bacterial populations are often large and, during infection, actively growing, which favours the emergence of mutational changes. Mutations leading to qualitative changes mainly occur in the genes encoding antibiotic targets, such as the ribosomal protein S12 for streptomycin. Mutations in chromosomal genes represent the only mechanism of antibiotic resistance in genera such as *Mycobacterium* or strictly intracellular pathogens, which do not exchange DNA. Resistance through chromosomal mutation is also becoming increasingly common in organisms causing clinical infections, and from 2000 to 2004 the occurrence of chromosomal resistance to nalidixic acid coupled with decreased susceptibility to ciprofloxacin increased from 14% to 20% in a study of over 20,000 isolates of *Salmonella enterica* per annum from cases of human infection in 10 European countries (Meakins *et al.*, 2008).

In many antibiotic-resistant variant strains of bacteria which cause clinical infections in man and animals, resistance results from horizontal acquisition of genes from other bacteria which may be ecologically and taxonomically distinct (Aminov and Mackie, 2007; Bennett, 2008).

The genetic mechanisms involved in the acquisition of antibiotic resistance genes from the resistome and the further dissemination by commensal and pathogenic bacteria include conjugation, transduction and transformation. Gene flow may be further enhanced by transposons and integrons (Bennett, 2008; Schlüter *et al*., 2007; D'Costa *et al*., 2007). As a consequence of these processes, a common pool of resistance determinants is shared by a diversity of bacteria as part of the horizontal gene pool (Schlüter *et al*., 2007). Horizontal

gene transfer will occur at measurable rates under conditions in which populations of suitable recipient bacteria build up and are in contact with the donors.

Conjugation is extremely relevant for transfer of genes between bacteria. It is the main process of horizontal transfer by which antibiotic resistance genes are acquired by bacteria. Conjugation implies the transfer between strains of self-transmissible plasmids or the mobilisation of other plasmids that are incapable of self-transmission, as well as the transfer of conjugative transposons (for a review, see Bennett, 2008). Plasmids frequently possess antibiotic resistance genes, notably within transposons and/or integrons, which favour the spread and expression of antibiotic resistance genes. Many plasmids and conjugative transposons have a broad host range, which in turn adds substantially to the spread of antibiotic resistance genes among bacteria. What determines host range has only been recently investigated (Bennett, 2008; Courvalin, 2008). Sublethal stresses (as encountered during food-processing) increase the inter- and intra-species horizontal transmission of plasmids containing antibiotic resistance genes by conjugation (McMahon *et al*., 2007a). Also, synergistic effects between biofilm formation and plasmid transfer by conjugation have been observed (Reisner *et al*., 2006).

In the context of food animal hosts, the respective contribution of dissemination of a resistant clone versus multiple transfer events has rarely been investigated. Also sublethal food preservation conditions (high/low temperature, osmotic and pH stress) can decrease antibiotic susceptibility in food-related pathogens (McMahon *et al*., 2007b). Such decreased susceptibility to antibiotics can be due to an induced activity of bacterial efflux pumps (Rickard *et al*., 2004), generation of genetic variability through mutation (Jolivet-Gougeon *et al*., 2000) or to an increase in plasmid copy numbers (Nandakumar *et al*., 2001).

In broilers, transfer of mobile elements between bacteria has made an important contribution in the prevalence of resistance (Smith *et al*., 2007). Because conjugation implies the exchange of DNA between viable bacterial cells it is not relevant for the transfer of antibiotic resistance genes from plants to bacteria.

Transduction is performed by bacteriophages, which carry along some of the host bacterial DNA when they replicate. Upon entering the bacterial cell, the DNA has to be stabilised either by forming an autonomously replicating element or by integration in the bacterial DNA. This integration depends mostly on homologous recombination and requires nucleotide-sequence similarity between the donor DNA and the recipient genome. Furthermore, most phages are host-specific or at least have a very restricted host range. Therefore, transduction is usually limited to the transfer of homologous genes among closely related bacteria. For these reasons, transduction would seem an unlikely candidate for efficient transfer of antibiotic resistance genes among unrelated bacteria. Transduction implies the transfer of DNA between viable bacterial cells and is, therefore, not relevant as a mechanism for the transfer of antibiotic resistance genes from plants to bacteria.

Transformation of bacteria includes the transfer of naked DNA from the environment into the bacterial cells (Kelly *et al*., 2008). This process requires the stabilisation of the DNA in the recipient cell. This stabilisation can occur by forming an autonomously replicating element or by integration in the bacterial DNA. The integration depends mostly on homologous recombination and requires nucleotide-sequence similarity between the donor DNA and the recipient genome. The mechanism of transformation has been only very rarely reported in connection with antibiotic resistance transfer between bacteria in nature. All documented

examples concern transfer between bacterial strains that share substantial DNA sequence homology. Genetic exchange by transformation has been reported between two closely-related strains of *Pseudomonas stutzeri,* rendering the bacteria resistant to rifampicin (Stewart and Sinigalliano, 1991). Natural interspecies transformation and subsequent recombination have been observed in the human pathogen *Streptococcus pneumoniae,* resulting in the appearance of mutant *pbp* genes and penicillin-resistant phenotype (Claverys *et al*., 2000). Transformation remains the only possible method of acquisition of genes by bacteria from plants.

In addition to conjugation, transduction and transformation, other less well recognised mechanisms of DNA uptake occur in nature (reviewed by Keese, 2008). These include vesicle-mediated translocation by a range of Gram-negative bacteria (vesicles with genes, *e.g.* antibiotic resistance and virulence genes bud from one cell and fuse with another cell), transfer by virus-like particles and mixing of entire genomes by cellular fusion occurring in multicellular bacteria.

2.1.2.2. Transfer frequencies of antibiotic resistance genes between bacteria

Table 3: Frequencies of antibiotic resistance gene transfer between bacteria by different processes of horizontal gene transfer

Transfer process Mobile element	Relatedness between donor and recipient	Experimental design	Frequency (transfer probability per CFU)
Conjugation Variable	Same genus	Variable	$10^{-3.46}$ (n=32, SD $10^{1.76}$) [a]
Conjugation Variable	Different genus	Variable	$10^{-6.33}$ (n=96, SD $10^{1.98}$) [a]
Conjugation Variable	Variable	In vitro, filter	$10^{-6.56}$ (n=84, SD $10^{1.68}$) [a]
Conjugation Variable	Variable	In vitro other	$10^{-3.46}$ (n=28, SD $10^{2.14}$) [a]
Conjugation Variable	Variable	*In vivo* (gastro-intestinal models)	$10^{-4.41}$ (n=16, SD $10^{2.3}$) [a]
Conjugation Plasmid pAMβ1	Variable	Variable	$10^{-5.90}$ (n=42, SD $10^{2.09}$) [a]
Conjugation Plasmid RP1	Variable	Variable	$10^{-1.80}$ (n=12, SD $10^{1.17}$) [a]
Conjugation Transposon Tn*916*	Variable	Variable	$10^{-7.21}$ (n=28, SD $10^{1.82}$) [a]
Transduction P1*kc*	*E. coli* (donor, recipient)	In vitro	$10^{-5.52}$ (n=3, SD=10^{-7}) [b]
Transduction T4GT7	*E. coli* (donor, recipient)	In vitro	$10^{-8.05}$ (n=1) [b]
Transduction EC10	*E. coli* (donor, recipient)	In vitro	$10^{-9.15}$ (n=3, SD=10^{-9}) [b]
Transformation Chromosomal	*C. coli* (donor, recipient)	In vitro Erythromycin	25°C: < 10^{-7} – $10^{-6.52}$; [c] 42°C: $10^{-2.52}$ – $10^{-6.15}$; [c]
Transformation Chromosomal	*C. coli* (donor, recipient)	In vitro Nalidixic acid	$10^{-2.7}$ – $10^{-3.4}$; [c] (temperature independent)

References: [a] Hunter *et al*. (2008); [b] Kenzaka *et al*. (2007); [c] Kim, *et al*. (2008a)

Transfer of antibiotic resistance genes between bacteria, especially across the bacterial species, takes place most frequently by conjugation (Table 3). Recently, a meta-analysis of published transfer rates of antimicrobial resistance genes was performed (Hunter *et al*., 2008).

A total of 34 papers were identified, of which 28 contained rates estimated in relation to either donor or recipient bacterial counts. The published transfer frequencies ranged from 10^{-2} to 10^{-9}. Generalized linear modelling was conducted to identify the factors influencing this variation. Highly significant associations between transfer frequency and both the donor ($P = 1.2 \times 10^{-4}$) and recipient ($P = 1.0 \times 10^{-5}$) genera were found. Also significant was whether the donor and recipient strains were of the same genus ($P = 0.023$) and the nature of the genetic element ($P = 0.0019$). The type of experiment, whether *in vivo* or *in vitro*, approached statistical significance ($P = 0.12$). Parameter estimates from a general linear model were used to estimate the probability of transfer of antimicrobial resistance genes to potential pathogens in the intestine following oral ingestion. The mean logarithms of these probabilities were in the range of [-7.0, -3.1]. These probability distributions are suitable for use in the quantitative assessment of the risk of transfer of antimicrobial resistance genes to the intestinal flora of humans and animals.

In comparison to conjugation, much lower frequencies were found for antibiotic resistance transfer by transduction (Table 3). Transfer frequencies of antibiotic resistance genes by transformation between the same naturally competent *Campylobacter coli* strain, was highly variable, dependent on the identity of the antibiotic resistance gene tested and the temperature (Table 3). Transformation frequency was not significantly different between microaerobic and aerobic conditions (Kim *et al*., 2008a).

2.1.2.3. Relevance of the data for the risk assessment

Antibiotic resistance genes are not only present in the antibiotic producer organisms but also in natural bacterial populations, where they are frequently carried on mobile genetic elements such as plasmids and transposons. Horizontal gene transfer between bacteria will occur at measurable rates in different environmental settings, including field, food and feed processing, human and animal gastrointestinal tract, either by conjugation, transformation or transduction. Conjugation is the most frequently reported mechanism of resistance transfer between different bacterial species and genera. Transduction and transformation appear to be limited to transfer between related bacteria or to the transfer of DNA fragments homologous to the recipient cell. It is not possible to define in quantitative terms the particular chain of events leading to the acquisition of specific antibiotic resistance gene by a specific pathogenic bacterium.

In conclusion, transfer of antibiotic resistance genes between bacteria occurs at measurable rates in different environmental settings. Conjugation is regarded as the main transfer mechanism, particularly for transfer between unrelated bacteria. No transfer of any chromosomally located antibiotic resistance gene has been observed by the process of natural transformation without the presence of homologous DNA in the recipient cell.

2.1.3. Effect of selection pressure on dissemination and maintenance of antibiotic resistance

2.1.3.1. Selection of resistance in natural environments

Selection for resistant bacterial phenotypes occurs in natural environments (reviewed by Aminov and Mackie, 2007). Antibiotic-producing bacteria are abundant in soil and there is evidence that such bacteria produce antibiotics in nutrient-enriched environments in soil. The

production of antibiotics is thought to represent a defence mechanism to protect organisms in the local environment against competing microorganisms, thereby being a key survival mechanism in nature.

An important role of antibiotic resistance genes in the soil antibiotic resistome is to protect organisms against naturally-occurring antibiotics (reviewed by Aminov and Mackie, 2007; Courvalin, 2008). The majority of antibiotics in use in clinical and veterinary medicine are produced in nature by microorganisms (*e.g.* streptomycetes produce many clinical therapeutic agents such as streptomycin), which contain the corresponding antibiotic resistance genes for self-protection. But how do bacteria cope with semi-synthetic antibiotics (*e.g.* amikacin) or entirely synthetic antibiotics (*e.g.* sulphonamides and fluoroquinolones), that are usually not encountered in nature? In such circumstances no natural reservoirs of corresponding resistance genes are considered to exist in the environment. It now seems apparent that endogenous genes that provide reduced susceptibility to certain antibiotics occur in the environment; mutations in the respective genes can lead to increased resistance. In the case of synthetic antibiotics such as the sulphonamides and trimethoprim, the genes encoding resistance have become associated with mobile genetic elements. The environment can thus act as a reservoir for both old and new resistance mechanisms. There is evidence that the resistance genes found within resistance gene clusters of the antibiotic producers have moved to other non-antibiotic-producing bacteria.

Metagenomic analyses, based on molecular detection of resistance determinants, allow the analysis of an extended bacterial population compared to conventional culturing methods. The expanding metagenomic studies have revealed an unexpected density of antibiotic resistance genes in the environment (D'Costa *et al*., 2007). The resistance mechanisms identified include inactivation of aminoglycoside antibiotics by phosphorylation and acetylation (Riesenfeld *et al*., 2004). The results indicate that soil bacteria are a reservoir of antibiotic resistance genes towards aminoglycosides with greater genetic diversity than previously accounted for. Even a remote Alaskan soil with no known exposure to antibiotics, harbours a great variety of resistance determinants (Allen *et al*., 2009) and even before the clinical use of antibiotics, antibiotic resistant bacteria were isolated (Wright, 2007). The ubiquitous nature of the great variety of antibiotic resistance genes can be explained by the fact that many of these genes are not just weapons against bacterial competitors but have other primary signalling functions (*e.g.* detoxification of metabolic intermediates, virulence and signal trafficking) (Martinez, 2008).

No pharmaceutically-produced antibiotic has been shown to circumvent the development of bacterial resistance, supporting the recent observation of the abundance of a great variety of resistance genes in the environmental bacterial population. To survive in the face of antibiotics, pathogens are continuously sampling the horizontal gene pool, in which they find natural resistances under adequate expression signals.

2.1.3.2. Dissemination and maintenance of the resistance genes influenced by selection pressure

There is increasing evidence that large amounts of antibiotics are released into the environment (soil and water) with waste water from hospitals, households and farms (Baquero *et al*., 2008; Jakobsen *et al.*, 2008; Moura *et al*., 2007; Thiele-Bruhn, 2003). These outflows constitute a selective pressure that can result in the selection of antibiotic-resistant environmental microorganisms. Information on the usage of antibiotics in both human and

veterinary medicine allows the identification of environments where strong positive selection for bacteria carrying resistance genes may be likely.

There are a number of cases that show correlation between antibiotic use and incidence of resistance (genes) among clinical isolates (for recent reports see, *e.g.* Monnet *et al*., 2004; Goossens *et al*., 2005; Hocquet *et al*., 2008). The use of antibiotics at sub-inhibitory concentrations represents a significant route for the selection and dissemination of antibiotic resistance genes (Aminov and Mackie, 2007; D'Costa *et al*., 2007 and references therein). Perhaps most important is the use of antibiotics for treatment of diseases in intensively-reared farm animals. These treatments can result in gut commensal and pathogenic bacteria acquiring resistance genes under selection. As a consequence of the way in which farm slurries are disposed of, such genes may then spread to soil bacterial communities (Agersø *et al*, 2006). Integrons with multiple resistance gene cassettes have been selected and disseminated in this way; many of these cassettes carry other genes, such as those conferring disinfectant resistance which have been co-selected in bacteria surviving in effluents and contaminated soils, thereby contributing to the maintenance and spread of the antibiotic resistance genes in the farm environment (Randall *et al*., 2005).

Exposure to antibiotics can enhance gene transfer (Courvalin, 2008 and references therein). For example, mitomycin C and ciprofloxacin derepressed the expression of genes necessary for transfer of an integrating conjugative element in *Vibrio cholera*e; this resulted in horizontal dissemination of the genetic element which confers resistance to streptomycin. Stress caused by certain antibiotics (*e.g.*, low concentrations of aminoglycosides) can induce the ability of *Streptococcus pneumoniae* to take up and integrate exogenous DNA.

Some knowledge gaps related to the current understanding of the composition and spatio-temporal dynamics of the resistome include an in-depth understanding of the natural reservoir of antibiotic resistance genes and their causal basis and role in natural bacterial communities not exposed to industrially produced antibiotics. In the great majority of cases, the particular chain of events leading to the acquisition of a specific antibiotic resistance gene by a specific pathogenic bacterium remains undescribed. The key role of selection by antibiotic usage in the development of resistance seems indisputable. Such understanding also emphasizes that gene transfer frequencies alone are of little value in predicting outcomes, and that the role of antibiotics in different environments and scenarios requires more attention.

2.1.3.3. Fitness cost of antibiotic resistance

Any gene transfer event that has a negative effect on the fitness of the bacteria will in general be quickly removed from a population, provided this population is subjected to periods of growth. Even near-neutral events are expected to be eliminated. In contrast, positively-selected events can become dominant, provided the selective force lasts long enough and bacterial growth is possible under such conditions.

It has been argued that the carriage of antibiotic resistance genes, may impose additional metabolic cost for the bacterial cell. As a consequence antibiotic resistance genes will be eliminated from the population once the selective pressure is removed. This is not always the case; *e.g.* a specific substitution in the *rpsL* gene responsible for high-level resistance to streptomycin in several enteric bacteria is a 'no-cost' mutation (Courvalin, 2008). There are also examples of compensatory evolution that reduce the biological cost and lead to the stabilisation of the resistant bacteria in a natural population (Aminov and Mackie, 2007 and references therein; Courvalin, 2008). There could also be co-selection of antibiotic resistance genes by other factors such as the presence of heavy metals, quaternary ammonium

compounds, stress (DNA-damaging agents), virulence factors *etc*. (references in Aminov and Mackie, 2007). Bean *et al*. (2005) demonstrated the persistence of sulphonamide resistance in *E. coli* from urine samples for over a decade after the almost complete withdrawal of these agents from clinical practice in the United Kingdom. Resistance determinants, particularly those encoded on highly mobile genetic elements can maintain themselves in the absence of direct selection (Enne *et al*., 2004).

2.1.3.4. Relevance of the data for the risk assessment

There is evidence that the environment acts as an important reservoir for antibiotic resistance genes conferring resistance to all natural and synthetic antibiotics. Once established, resistant organisms may persist and may spread widely, a process facilitated by antibiotic usage. Antibiotic resistance genes may impose additional metabolic cost for the bacterial cell. Such resistance can be maintained in an antibiotic-free environment.

There is no experimental evidence linking antibiotic resistance marker genes of GM plants to the environmental abundance of antibiotic resistances or their genes.

In conclusion, the environment acts as a reservoir for genes giving rise to resistance to natural and synthetic antibiotics. This is due to the genesis of resistance as a natural event. Selection for resistant bacterial phenotypes occurs in natural environments as a defence mechanism. The use of antibiotics is a key factor in the selection and dissemination of antibiotic resistance genes in the immediate environment. Resistance genes can move between bacteria by a variety of methods, both within and outside closely-related bacterial species.

2.2. Distribution and abundance of antibiotic resistant bacteria in the environment and in feed and food sources

Bacteria resistant to different aminoglycosides, including the ones relevant in the context of antibiotic resistance markers in GM plants (i.e. kanamycin/ neomycin, and streptomycin/ spectinomycin) have been isolated from soils, surface waters, sewage sludge, manure and other environmental samples including faeces (Heuer *et al*., 2002; Seveno *et al*., 2002; Van Overbeek *et al*., 2002).

It is important to consider the release of antibiotics into the environment as a risk factor when considering circumstances that may lead to an increase in antibiotic resistance and antibiotic resistance encoding genes.

In food products, both commensal and pathogenic bacteria resistant to different aminoglycosides have been detected (EFSA, 2008a). Antibiotic-resistant *Salmonella* and *Campylobacter* involved in human disease are mostly spread through food. *Salmonella* is a prominent pathogenic bacterium in contaminated poultry meat, eggs, pork and beef, environment and direct animal contact, and *Campylobacter* in contaminated poultry meat. Cattle are the major reservoir for verotoxigenic *Escherichia coli* (VTEC), and resistant strains may colonize humans *via* contaminated bovine meat or environment contaminated by bovine faeces more commonly than *via* other foods. Direct contact with colonised animals or their direct environment is also a source of meticillin-resistant *Staphylococcus aureus* (MRSA). For further information on this topic consult BIOHAZ Opinion (EFSA, 2009). Contaminated food can also be a source of human infections with antimicrobial-resistant *Shigella* spp. and *Vibrio* spp.

It is considered that the occurrence of resistant pathogenic bacteria in food-producing animals or foods is a consequence of selective pressure by antimicrobial usage in veterinary and human medicine, although this relationship can not currently be quantified.

2.2.1. Methods to detect antibiotic-resistant bacteria from environmental and food samples

A considerable amount of information on the prevalence of antibiotic-resistant bacteria has been gathered from studies in which microbial consortia from environmental matrices were cultivated in the presence of specific antibiotics, under laboratory conditions. The prevalence of antibiotic-resistant bacteria within the non-culturable majority of environmental bacteria remains unknown. In addition to the culture-based methods, molecular methods have therefore been developed and applied to study the environmental prevalence of antibiotic-resistant genotypes or resistance genes without the need to cultivate bacteria. These methods include the direct detection of antibiotic resistance genes by PCR or by hybridization with a specific gene probe as well as capturing plasmids transferred from non-cultured bacteria into well-defined culturable recipients such as *Escherichia coli* K-12 derivatives using biparental or triparental mating.

Neither culture-based or molecular methods nor their use in combination can give a complete picture about the actual abundance of antibiotic-resistant bacteria and antibiotic resistance genes in the environment or of the contribution of bacterial genes derived from plants, as the latter genes are indistinguishable from those already present in the bacterial population. Recent metagenomic DNA sequencing experiments have demonstrated that the diversity and abundance of antibiotic resistances is likely to be larger than suggested by most currently applied methods (Riesenfeld *et al*., 2004). New high-throughput DNA sequencing methods should contribute greatly to the characterization of the antibiotic resistance gene pool.

2.2.2. Detection of kanamycin/neomycin-resistant isolates and *aph(3')*-IIa (or *npt*II) gene

2.2.2.1. Kanamycin/neomycin resistance phenotype

Kanamycin and neomycin resistance phenotypes are widespread in habitats such as soils, manures, and (waste) waters with up to 10^5 resistant bacteria per gram of soil in many soils. Furthermore, resistance to kanamycin has been found in bacteria from clinical, animal husbandry and food-processing environments. For instance, such resistance has been found in bacteria from poultry in the USA (Kelley *et al*., 1998). In Norway, resistance to neomycin has been found in *Enterococcus faecalis* from poultry faeces in 2000-2003 (Wögerbauer, 2007; Tables 18/19). Kanamycin resistance were also found in *E. coli* from pork (Wögerbauer, 2007; Table 20). Recent reports also indicate aminoglycoside resistance in particular bacterial strains (Schmitz *et al*., 1999; Gibreel *et al*., 2004; Hauschild *et al*., 2007). Examination of enterococcal isolates from wild boars in Portugal revealed considerable numbers of kanamycin-resistant isolates, mediated by aph(3')-IIIa, as well as streptomycin resistances (Poeta *et al*., 2007). In poultry, a high incidence of resistance to kanamycin (30%) has been reported, reflecting different selection pressures active in different environments (Novais *et al*., 2005). Resistance to neomycin has been reported for *E. coli* from pigs (Brun *et al*., 2002). Also kanamycin resistance was observed in enterotoxic, non-enterotoxic and commensal *E. coli* isolates from pigs (Travis *et al*., 2006). Five percent of the pseudomonads from poultry

were resistant to kanamycin and 10% to neomycin (usually part of a multi-resistance locus; Kelley *et al*., 1998) and kanamycin resistance was observed in a study of soil and water environments (Esiobu *et al*., 2002). In contrast resistance to kanamycin was uncommon (< 2%) in isolates of *Salmonella enterica* from cases of human infection in 10 European countries from 2000 – 2004 (Meakins *et al*., 2008).

Overall, the available data indicate that kanamycin and neomycin resistance are present in bacterial populations of a range of habitats, but there is high variability in the incidences. Habitats with low and high resistance levels have been reported, presumably reflecting the effects of variable selection pressures. Also, different bacterial species may differ substantially in their susceptibility to these antibiotics.

A recently identified threat is that of the emergence of 'extensively drug-resistant' (XDR) tuberculosis, defined as tuberculosis caused by *Mycobacterium tuberculosis* strains that are resistant to at least rifampicin and isoniazid (MDR-TB), in addition to resistance to fluoroquinolones and at least one of three injectable second-line drugs, namely amikacin, kanamycin or capreomycin (Jain and Mondal, 2008; Jassal and Bishai, 2009). Recent studies have described XDR-TB in all continents and the world-wide prevalence is estimated to be between 6% and 9% in countries studied (Shah *et al*., 2007).

2.2.2.2. Distribution of *aph(3')*-IIa (*npt*II) gene

The *npt*II gene was first discovered as the kanamycin/neomycin resistance determinant of transposon Tn*5*, which has a broad host range across the proteobacteria. The gene was thus originally present in a clinical setting and has later been found outside clinical settings. Indeed, several clinical strains with kanamycin/neomycin resistance have been found to carry the *npt*II gene as the resistance determinant. In particular Gram-negative bacteria such as *Pseudomonas* spp., *Citrobacter freundii, Enterobacter cloacae, Aeromonas* spp. and *Escherichia coli* have been implicated (Alvarez and Mendoza, 1992). Two key studies have provided additional data on the distribution of the *npt*II gene in open environments (Leff *et al*., 1993; Smalla *et al*., 1993). Smalla *et al*. (1993) analyzed environmental (soil, water, manure) samples from the Netherlands and Germany. Leff *et al*. (1993) examined streams and other environments in the USA. Whereas evidence for the occurrence of *npt*II genes in soil was scarce, the gene was shown to be quite abundant and functional in manure, sewage and water samples. In a recent study, Zhu (2007) analyzed samples from Canada for the prevalence of the *npt*II gene. The study demonstrates the presence of *npt*II sequences with sequence homologies >97.9% to the Tn*5* gene in river microbial communities. The abundance varied over time, ranging up to 4.36×10^6 copies per litre of water.

In summary, the available data indicate that a pool of *npt*II-carrying bacteria occur in and outside hospital-associated environments. The data also indicate large environmental fluctuations in the abundances.

2.2.3. Detection of streptomycin/spectinomycin resistant isolates and *ant(3'')*-Ia (*aad*A) gene

2.2.3.1. Streptomycin resistance phenotype

Many studies have shown that the streptomycin resistance phenotype is ubiquitous in soil, water, animal, food and clinical environments. For example, more than 30% of the bacteria from farm soil were resistant to streptomycin (Srinivasan *et al*., 2008). Streptomycin resistance was the most frequent resistance in a collection of more than 1000 *Escherichia coli* strains from the faeces of domestic and wild animal as well as human septage and surface water (Sayah *et al*., 2005). Streptomycin resistance was also frequent in *Salmonella* isolated from healthy swine (Martin *et al*., 2008) and diseased animals (Zhao *et al*., 2005, 2007), as well as in Shiga toxin-producing *E. coli* from human and animals (Singh *et al*., 2005). In Great Britain, streptomycin resistance (among other antibiotic resistances), was frequent in *E. coli* from pigs but rare in isolates from cattle and sheep (Enne *et al*., 2008). Streptomycin-resistant bacteria were also found in drinking water (Walia *et al*., 2004). In a study of soil and water environments a widespread incidence of streptomycin resistance was found (Esiobu *et al*., 2002). High-level resistances to streptomycin (and gentamicin) were found in isolates in another study of Poeta *et al*. (2006). Moderate incidences of streptomycin-resistant bacteria were found in sewage effluent in Chile (Silva *et al*., 2005). *Klebsiella pneumoniae* isolates from a turkey farm in Oklahoma were all resistant to streptomycin as well as to kanamycin (Kim *et al*., 2005). In a trans-European survey study, evidence for the prevalence of streptomycin resistance genotypes (different genes) was found in environments ranging from soils to manures to bulk waters (Van Overbeek *et al*., 2002).

Salmonella Typhimurium definitive phage type (DT) 104 is a multiresistant phage type with almost global epidemicity (Threlfall, 2000). The organism has caused outbreaks in many countries throughout the world, with a variety of food associations (Molbak *et al*., 1999; Threlfall, 2000). The strain is typically penta-resistant [ampicillin, chloramphenicol/florfenicol, streptomycin/spectinomycin (encoded by an *aad*A gene), sulphonamides and tetracyclines (ACSSpSuT)], with the resistances encoded within a mobile genetic element designated *Salmonella* Genomic Island-1 (SGI-1). SGI-1 with *aadA*2 has subsequently also been identified in other Typhimurium phage types, as well as in at least 10 other *Salmonella* serotypes worldwide, including Agona, Albany, Newport and Paratyphi B. Resistance to streptomycin is also common in *E. coli* from beef, poultry meat and pork.

2.2.3.2. Distribution of *ant(3'')*-Ia (*aad*A) gene

The *aad*A gene, originally identified in the plasmid R538-1 of *E. coli*, has also been found in soils. Additionally, *aad*A genes have been found in Siberian permafrost soil sediments (Mindlin *et al*., 2008) and in farm soils (Srinivasan *et al*., 2008). Approximately one third of the streptomycin-resistant bacteria in the faeces of some dogs and cats in Portugal carried the *aad*A gene (Costa *et al*., 2008). The *aad*A gene has also been recently reported in streptomycin-resistant *E. coli* (Dolesjká *et al*., 2008; Kadlec and Schwarz, 2008), *Salmonella* (Martin *et al*., 2008; Zhao *et al*., 2007; Antunes *et al*., 2006; Meakins *et al.,* 2008) and in Shiga toxin-producing *E .coli* from food animals and humans. Based on these data the *aad*A gene appears to be widely distributed in the environment as well as in food animals and humans.

2.2.4. Relevance of the data for the risk assessment

Habitats such as soils, waters, manure and wastewater provide a reservoir of environmental bacteria that possess antibiotic resistance determinants, including those conferring kanamycin- neomycin- and streptomycin- resistance phenotypes. Furthermore, metagenomic analysis allowing the analysis of total bacterial populations (including non-culturable bacteria), has demonstrated that these resistant determinants were present in all environments investigated. From this reservoir, antibiotic resistance determinants may be selected and further disseminated to the human environment.

Resistance to kanamycin, neomycin and streptomycin has been found in bacteria from clinical, animal and food-processing environments. Environmental fluctuations in the abundance are recognised, presumably reflecting the effects of variable selection pressures. The resistance genes are increasingly found on mobile genetic elements and may spread across bacterial communities. This has implications for medical and veterinary practice, as a subset of resistant organisms can be selected under clinical conditions coupled with intensive antibiotic selective pressure.

In conclusion, recent metagenomic analyses of total bacterial populations (including non-culturable bacteria), have demonstrated that resistance determinants for kanamycin and neomycin and streptomycin have been detected in all the environments investigated. The *npt*II and *aad*A antibiotic resistance genes and their phenotypes have a wide distribution, albeit at different frequencies, in different species, isolates and different environments, in naturally occurring bacteria.

3. Antibiotics with relevance to this opinion: usage and clinical importance

3.1. Usage patterns of aminoglycosides relevant to this Opinion

Antibiotics are used both in human and veterinary medicine to prevent and cure bacterial infections. In animals they have also been used to enhance growth and feed efficiency; the authorization to use antibiotics as growth promoters has been withdrawn in the EU in 1998 (EC, 2003; EC, 1998). In horticulture streptomycin and tetracycline are used to some extent in plant protection against bacterial infections. For several purposes the same antibiotics have been used.

Arguably the most important factor influencing the emergence and spread of antimicrobial resistance is the use of antimicrobial agents in different hosts, followed by the spread of resistant bacteria and resistance genes between hosts of the same or of different species (SSC, 1999). In the human, veterinary and horticultural spheres there is a variety of ways in which antimicrobials are dispensed and applied. In human medicine, antimicrobials are widely used for therapy and prophylaxis both in hospitals and in the community, under varying levels of supervision. Likewise, the same antimicrobial agents are widely used in animals and aquatic species bred for food production. Such usage is for therapeutic treatment, prophylaxis and in some countries outside the EU, for growth promotion. Antibiotics are also used for therapy and prophylaxis in companion animals, also under varying degrees of supervision. Oral medication of large groups of animals is particularly likely to favour emergence of and selection for antimicrobial resistance. In primary production, conditions such as high density and/or poor infection control may facilitate the spread of resistant bacteria.

Likewise, the release of antibiotics into the environment *via* effluents from hospitals and the community in general (see 2.2. above) is a risk factor that may lead to an increase in antibiotic resistance and antibiotic resistance conferring genes.

Antimicrobials are grouped into classes on the basis of chemical structure and mode of action. Most antimicrobials used for the treatment of animals belong to classes that are also used in human medicine. A list of antimicrobial classes was compiled by WHO in 2007 (the WHO Expert Group on Critically Important Antimicrobials for Human Health), giving examples of substances used for the treatment of infections in humans. Antimicrobials listed as 'Critically Important' in the WHO list are characterised as both 1.'sole therapy or one of few alternatives to treat serious human disease', and 2: 'antimicrobials used to treat diseases caused by organisms that may be transmitted *via* non-human sources or diseases caused by organisms that may acquire resistance genes from non-human sources'. Antimicrobials classified as 'Highly Important' meet either criteria 1 or criteria 2. Of relevance to this Opinion, the aminoglycosides listed as 'Critically Important' included amikacin, gentamicin, netilmicin, tobramycin, gentamicin, and streptomycin, whereas kanamycin, neomycin and spectinomycin are listed as 'Highly Important'.

The WHO Expert Group also recognized the need for more data on antimicrobial resistance attributable to the non-human use of antimicrobials, data on factors that lead to the development and spread of resistance in various pathogens in animals and humans, and better data on the benefits of antimicrobial usage in both human and veterinary medicine (WHO, 2007).

The World Animal Health Organisation (OIE) has similarly developed and adopted a list ranking the importance of different antimicrobials for animal health (OIE, 2007).

In both human and veterinary medicine there is concern that the clinical benefits associated with the above antimicrobial agents, and specifically kanamycin, neomycin and streptomycin, when used as all but a last resort in the treatment of infectious diseases, will be diminished as a result of the acquisition of resistance, for whatever reason, by the causal bacteria.

Currently there is evidence of a global pandemic of antibiotic resistance. Examples include multiresistant *S.* Typhimurium DT 104 (see above), vancomycin-resistant enterococci, ESBLs (extended-spectrum beta-lactamase-producing enterobacteriaceae), MRSA (meticillin-resistant *Staphylococcus aureus*), VRSA (vancomycin-resistant *Staphylococcus aureus*), multiresistant *Clostridium difficile* and extensively drug-resistant (XDR) TB. The antibiotics in use are usually improved versions of earlier ones rather than truly new ones, which would be based on biological pathways for attacking bacteria. Therefore great care requires to be taken to preserve the efficacy of all antibiotics, including those not currently in use for a particular indication.

Aminoglycosides are poorly absorbed through the gastrointestinal tract, and about 97% of an orally administered dose is excreted unchanged into the environment. The use of these antibiotics is also limited by their potent dose-dependent acute ototoxicity (vestibular damage, hearing loss and tinnitus) together with nephrotoxicity (kidney toxicity is the major limitation to their use in human medicine). The indicated uses in human and veterinary medicine are: 1) topical uses for *e.g.* skin, eye and ear infections (humans and animals); 2) oral uses, which are restricted to gut irrigation and encephalopathy in humans (neomycin) and to treatment of diarrhoea in farm animals; 3) aerosol administration for respiratory infections in humans and

animals; 4) parenteral use of some aminoglycosides (amikacin, gentamicin, netilmicin, tobramycin and kanamycin) for serious and life threatening infections in humans.

3.2. Antibiotic usage and clinical importance

In human medicine, consumption data have been made available by ESAC (European Surveillance of Antimicrobial Consumption), made available through EMEA. Although aminoglycoside consumption data reflect the importance of many of these compounds individually and overall for aminoglycosides, data also indicate the limited systemic use of kanamycin and neomycin. Additional information from EU regulatory agencies confirm that these compounds are limited in systemic use to important indications like second line treatment for multiresistant tuberculosis (kanamycin) and in gut irrigation in, for example, encephalopathy (neomycin). There is also considerable topical use of these antibiotics, which is not reflected by the ESAC data.

Of the antibiotics of relevance to this Opinion, streptomycin is indicated for the treatment of individuals with moderate to severe infections caused by bacteria shown to be susceptible to the antibiotic and which are not amenable to therapy with less potentially toxic agents. Infections which are treated with streptomycin include *Yersinia pestis* (plague), *Francisella tularensis* (tularemia), *Brucella,* and *Haemophilus influenzae*. Spectinomycin is used for the treatment of gonorrhoea, and kanamycin is used as a second-line antimicrobial agent for the treatment of multiple drug-resistant tuberculosis. Neomycin is used for preoperative preparation of the bowel as adjunctive therapy as part of a regimen for the suppression of the normal bacterial flora of the bowel.

During the last few years several EU countries have set up monitoring programs for measuring antibiotic usage in animals. The data have been collected between 2004 and 2007. Except in Sweden tetracyclines are the most frequently used products (48-60%) followed by β-lactams (10-29%). Aminoglycosides constitute between 2 and 7 percent of the total usage. In the United Kingdom the consumption of aminoglycosides has been reported for specific compounds: the numbers in 2005 were 6000 kg active compound of streptomycin, 6000 kg of neomycin and kanamycin and 11000 kg of other aminoglycosides. According to the 1999 report of EMEA, aminoglycosides for veterinary use represented 154 tons, which was approximately 4% of the therapeutic veterinary market. The oral use of aminoglycosides in veterinary medicine in the EU included kanamycin, neomycin, paromomycin, gentamicin, apramycin, streptomycin and spectinomycin.

Antibiotics affected by the kanamycin resistance gene* aph(3')-*IIa* (npt*II gene)

The *aph(3')*-IIa gene confers resistance to neomycin and kanamycin but not to other aminoglycosides of clinical use. The *aph(3')*-IIa gene confers slightly reduced susceptibility to amikacin for *E. coli* (Appendix A, Table A1). However, amikacin is a poor substrate for APH(3')-IIa enzyme due to its hydroxyaminobutyryl side chain.

***Human use* - Kanamycin** is rarely used systemically today due to its severe side effects although this antibiotic remains a recognized second line choice in conditions of infections with multiple drug resistant (MDR) *Mycobacterium tuberculosis* (MTB). Aminoglycoside resistance in *M. tuberculosis* results from a mutation causing alterations in the antibiotic target molecule within the mycobacterial cell; thus chromosomal resistance, and not the transfer of antibiotic resistance gene [such as *aph(3')*-IIa or *ant(3'')*-Ia], is the only identified

mechanism resulting in resistance to kanamycin, streptomycin and several other antibiotics (Goldstein *et al*., 2005 and references therein). The increasing occurrence worldwide of isolates of MTB with resistance to second-line antibiotics such as kanamycin (XTB) is of global concern (see Section 2.2.2.1). **Neomycin** is poorly absorbed from the gastrointestinal tract, and is nephrotoxic and ototoxic. The use of neomycin in human medicine is limited to topical applications and gut irrigation/encephalopathy (see above). By killing bacteria in the intestinal tract, it keeps ammonia levels low and prevents hepatic encephalopathy, especially prior to gastrointestinal surgery. Both kanamycin and neomycin are listed as 'highly important' in the 2007 WHO list (see above).

Veterinary use - Aminoglycosides, as a group, is a class of antibiotics critically important for veterinary medicine and animal production (OIE, 2007). As in human medicine resistance continues to increase to the alternative drugs. Therefore, the importance of neomycin and kanamycin and future derivatives of these drugs can be expected to increase. In veterinary medicine this could, for example, mean therapies of neonatal diarrhoea in piglets and treatment of multi-resistant enteric gram-negative infections.

No established clinical use - Geneticin (G418, geneticin) is only used for *in vitro* experimentation, *e.g*. as a selective agent for eukaryotic GM cells. For ribostamycin, butirosin, lividomycin, neamine and sisomycin there are no reported human use outside a few small clinical trials (butirosin, sisomycin) although their spectrum of activity would indicate advantages over other agents against, for example, *Pseudomonas aeruginosa*. Paromycin has been used in the treatment of various intestinal infections like cryptosporidiosis in man but has no established role in the treatment of infectious diseases in man.

Antibiotics affected by the streptomycin resistance gene* ant(3'')-*Ia* (aad*A gene)

Streptomycin was the first aminoglycoside to be discovered, in 1943. It was the first chemotherapeutic agent to be effective against *Mycobacterium tuberculosis* (Greenwood, 1995). Streptomycin is vestibulotoxic and cochleotoxic and has mostly been replaced by newer aminoglycosides. It is used in human medicine to a limited extent only (WHO, 1993). Streptomycin is still indicated as a component of a regimen used to treat tuberculosis, plaque and brucellosis and, in combination with a beta-lactam agent or glycopeptides, for treatment of enterococcal endocarditis with high-level gentamicin (but not streptomycin) resistance. Streptomycin is also used as a plant protection product in agriculture, mainly in certain horticultural crops, in the USA and Japan. In Europe it is only allowed for emergency use on crops.

3.3. Relevance of the data for the risk assessment

Kanamycin is categorized by the WHO Expert Group on Critically Important Antimicrobials for Human Health as a as 'Highly Important Antimicrobial'. It is used as a second-line drug for the treatment of infections with multiple drug-resistant tuberculosis (MTB). The increasing occurrence worldwide of "extensively drug-resistant" (XTB) isolates of MTB with resistance to second-line antibiotics such as kanamycin is a cause for global concern. The *npt*II gene has not been implicated in such resistance. Notwithstanding the relatively limited oral use of kanamycin and neomycin these compounds remain important both in human and veterinary medicine. When used they contribute to the selection and dissemination of relevant

antibiotic resistant bacteria present in the exposed environment. The impact could be a compromise of therapeutic use of those antibiotics.

The WHO Expert Group on Critically Important Antimicrobials for Human Health has also categorised streptomycin as a 'Critically Important Antimicrobial', and spectinomycin as a 'Highly Important Antimicrobial'.

Conclusions based on present state of scientific knowledge

- Available studies have not demonstrated transfer of antibiotic resistance marker genes from GM plant material to bacteria in the natural environment, nor has such transfer been detected from the existing background. This conclusion is supported by inferences from bioinformatics studies. The sensitivity of the field and laboratory studies cited is a limiting factor in the detection of transfer.
- Laboratory and field studies have demonstrated that horizontal gene transfer from GM plants into bacteria has only been observed when facilitated by the existence of DNA identity between the transgene and recipient bacterium. Recovery of the plant DNA by naturally occurring bacteria has not been demonstrated even when bacteria are exposed to DNA naturally released from plant tissues.
- The key barrier to stable uptake of antibiotic resistance marker genes from GM plants by bacteria is the extent of DNA sequence identity.
- Current scientific data show that source-independent uptake of free DNA molecules into the cytoplasm of naturally occurring competent bacteria may occur. Where efficient mechanisms for integration into bacteria are absent, stabilization of acquired traits is not detected.
- No transfer between bacteria of any chromosomally located antibiotic resistance gene has been observed by the process of transformation without homologous DNA in the recipient cell.
- Transfer of antibiotic resistance genes between bacteria occurs at measurable rates in different environmental settings. Conjugation is regarded as an important mechanism, particularly for transfer between unrelated bacteria. The environment is an important source of antibiotic resistance genes as regards natural and synthetic antibiotics. This is due to the genesis of resistance as a natural event. Selection for antibiotic-resistant bacterial phenotypes occurs in natural environments as a defence mechanism.
- The presence and use of antibiotics are key factors in the selection and dissemination of antibiotic resistance genes. Such resistance genes can move between bacteria by a variety of methods.
- Recent metagenomic analyses of total bacterial populations (including non-culturable bacteria) have demonstrated that resistance determinants of kanamycin, neomycin and streptomycin are present in all environments investigated.
- The *npt*II and *aad*A antibiotic resistance genes and their phenotypes have a wide distribution, albeit at different frequencies, in different species, isolates and different environments, in naturally occurring bacteria.
- Neither culture-based or molecular methods nor their use in combination can give a complete picture of the actual abundance of antibiotic resistant bacteria and antibiotic resistance genes in the environment or of the contribution of bacterial genes derived

from GM plants as these genes are indistinguishable from those already present in the bacterial population.

- Kanamycin and neomycin are categorized by the WHO Expert Group on Critically Important Antimicrobials for Human Health as a 'Highly Important Antimicrobial'. Kanamycin is used as a second-line drug for the treatment of infections with multiple drug-resistant tuberculosis (MTB). The increasing occurrence worldwide of "extensively drug-resistant" (XTB) isolates of MTB with resistance to second-line antibiotics such as kanamycin is a cause for global concern. The *npt*II gene has not been implicated in such resistance.
- The above WHO group has also categorised streptomycin as a 'Critically Important Antimicrobial', and spectinomycin as a 'Highly Important Antimicrobial'.

4. Risk assessment

4.1. Introduction

The general principles for risk assessment of GM plants as expressed in the Directive 2001/18/EC of the European Parliament and of the Council (EC, 2001) and in the guidance document of the GMO Panel (EFSA, 2006a) have been followed here. The following issues have been specifically addressed: 1) Identification of characteristics which may cause adverse effects (hazard identification); 2) Evaluation of the likelihood of functional gene transfer and evaluation of the potential consequences of the gene transfer, if it occurs (hazard characterisation) (Chapters 2 and 3); and 3) Evaluation of the overall potential risk posed by the presence of antibiotic resistance marker genes in GM plants.

The underlying question to be addressed is to what extent if any, cultivation of GM plants in which bacterial antibiotic resistance genes have been introduced as selectable markers contributes to the development of antibiotic-resistant bacteria of clinical importance.

There are a number of hypothetical routes through which the antibiotic resistance marker genes from GM plant material could be transferred to human pathogenic bacteria (see also Nap *et al*., 1992):

Environment: Transfer from damaged or decaying plant material to the indigenous microbiota of soil, in water environment, in wild animals (including grazing birds and mammals, earthworms, pollinating and other arthropods) and in or on plants. These communities may include some human or animal pathogenic bacteria (*e.g*., *Pseudomonas aeruginosa*, *Enterobacteriaceae*) or non-pathogenic bacteria, as the first recipients of antibiotic resistance genes can move to other habitats (*e.g*. clinical environment) and transfer from there to pathogens.

Food and feed processing environment: Transfer from plant material to microbiota during food and feed processing. The microbiota includes pathogenic and non-pathogenic bacteria present in the unprocessed food and feed product. They can also enter the food and feed chain by contamination during and after processing. In some processes bacteria are deliberately added as starter cultures for fermentative processes or as probiotics. Different food and feed processing methods have different influences on bacterial viability, leading to dead, sub-lethally injured or stressed bacterial cells. These processes also influence the intactness of the

bacterial cells, in some cases leading to the liberation of free bacterial DNA into the food and feed environment.

Food-producing animals: Consumption of GM feed, release of antibiotic resistance marker genes and other DNA into the gut by digestion, transfer to indigenous intestinal bacteria, including potential human pathogens, or from commensal bacteria to human pathogens existing in other environment, as indicated above. Transfer to human by ingestion of animal-derived food products.

Pet animals: Consumption of GM pet food, release of antibiotic resistance marker genes and other DNA into the gut by digestion, transfer to indigenous intestinal bacteria. Transfer to humans or food animals by direct contact with pet animals.

Humans: Consumption of uncooked/cooked GM-derived food, followed by transfer to intestinal bacteria and to bacteria pathogenic to humans (either in the intestinal tract or in other anatomical locations); particular vulnerable groups may include persons on antibiotic therapy and those with gastrointestinal disease.

4.2. Identification of characteristics which may cause adverse effects (hazard identification)

The hazard is regarded as the antibiotic resistance marker gene(s) introduced in the GM plants. The event potentially leading to adverse effect is the transfer of these genes from the GM plants into bacteria which inhabit humans, animals or which occur in the wider environment. The proteins encoded by the antibiotic resistance genes inherently have the capacity to inactivate specific antibiotics and thus provide resistance to bacteria against these antibacterial agents. In the case of human, animal or plant pathogens, this may lower the efficacy of a chosen antibiotic therapy. The ultimate adverse effect would thus be to compromise prophylactic or therapeutic medical, veterinary, or plant protection treatments.

4.3. Evaluation of the likelihood of occurrence of functional gene transfer

Horizontal gene transfer between bacteria is a common event. This has been demonstrated experimentally, both *in vitro* and *in vivo* (Chapter 2.1.2) and also predicted to have occurred in an evolutionary scale, using genomic databases.

How likely is the functional transfer of antibiotic resistance marker genes of bacterial origin from a GM plant to a bacterium? To answer this question it is necessary to distinguish between the relevance of each step in the causal pathway of a gene transfer event, based on available data, as well as to consider their intrinsic limitations, as listed below:

(i) availability of DNA with intact antibiotic resistance marker genes released from plant material by the decay processes in the environment or by digestion processes in the buccal cavity and the rest of the alimentary tracts of humans and animals (Chapter 2.1.1.1)

DNA from plants is released into the environment during cultivation and after harvest, as a result of lysis and senescence of plant material. The persistence of extracellular DNA in the environment is influenced by a number of biotic and abiotic factors, which favour DNA protection or induce DNA degradation. Persistence of DNA from transgenic plants in the field can vary from several weeks to several years. GM plant material used in food products is often subject to a variety of processing and storage regimes. Food processing and extraction of food and feed ingredients can physically and chemically damage and degrade DNA, but

can in some cases result in the release of intact DNA into the local, *i.e.* food or feed environment. The persistence in the digestive tract (from mouth to colon) of DNA in food depends on enzymatic degradation and on chemical degradation by low pH conditions. Food-derived DNA would be degraded more slowly than naked DNA; intact genes have been reported to be still in stomach contents but not in the large intestine.

(ii) competence of bacteria to undergo natural transformation in the habitats into which intact DNA is potentially released from the GM plant (Chapter 2.1.1.1)

Some bacterial species possess highly evolved processes that allow them to take up DNA from the environment. The development of 'competence' is a regulated process that may depend on particular environmental circumstances and seems to be widely distributed among very different phylogenetic groups. Competence development requires that bacteria are in a metabolically active state. Several researchers hypothesize that competence would be enhanced in bacteria living in aggregates on the different surfaces of the plant. On the other hand, *in situ* soil conditions are not always conducive to metabolically active bacteria, due to nutrient limitation. Soil is heterogeneous and composed of a multitude of nutrient-rich microhabitats, such as the plant rhizosphere, the residuosphere (the decaying material of animal or plant origin), and the pathosphere, which could foster competence development. In the gastrointestinal tract, inhibitory effects of rumen fluid and ovine saliva on the development of competence have been described. In relation to food products, the development of competence and transformation has been shown for *Bacillus subtilis* in milk.

(iii) Availability for integration of the intact antibiotic resistance marker gene after uptake by the recipient cell (Chapter 2.1.1.1)

Once foreign DNA enters the bacterial cell, it is susceptible to degradation by nucleases.

(iv) Integration of antibiotic resistance marker genes into the bacterial genome (chromosome or plasmids) is a prerequisite for their stable inheritance (Chapter 2.1.1.3)

Stabilisation of plant DNA in bacteria can occur in two ways: (a) by forming an autonomously replicating element when an origin of replication has been co-transferred to the GM plant and the replication functions are present in the recipient cell, or (b) by integration of plant sequences into the bacterial DNA by homologous recombination. Absence of homologous sequences or origins of replication are the major barriers to horizontal gene transfer by transformation. Non-homologous or illegitimate recombination is reported as a very inefficient process. Shortening the length of the identical sequence on both sides of the gene to about 300 bp and 100 bp results in about 10^4 and 10^7 reduction in transformation frequency, respectively. Homology-facilitated illegitimate recombination is reported but the frequencies of transformation *in vitro* were up to six orders of magnitude lower than those determined by transforming these strains with entirely identical donor DNA.

Transfer of an antibiotic resistance gene of bacterial origin from a plant to bacteria, if it is to occur, requires to meet comparable conditions. If the antibiotic resistance gene is already present in the recipient bacterium, homologous recombination will not have any impact.

On a theoretical basis, it can be anticipated that the gene context of the antibiotic resistance marker gene in the plant (*e.g.* the co-transfer of a bacterial origin of replication, short sequences recognised by transposases and integrases or bacterial vector sequences) may influence the stabilization of the transformed DNA. Based on the experimental transformation frequencies, it can be concluded that only bacterial vector sequences with a sufficient length of identity and located at both sides of the ARM gene would have a significant impact on the transformation frequency in comparison with constructs where no homology is present. Also

the presence of an origin of plasmid replication would only enhance the stabilisation of transforming DNA if sufficient identity is present, allowing circularisation of the linear transformed DNA in the recipient cell. Above this, replication would only be possible in recipient bacteria containing trans-acting replication functions, a probability depending on the presence of the plasmid in the recipient bacterial cell.

Also the source of DNA influences the transformation frequency. Transformation with purified plant DNA in comparison with plasmid DNA decreases the transformation frequency.

(v) expression of phenotypic resistance as a result of presence of the antibiotic resistance gene in the bacterium (or in subsequent recipients) (Chapter 2.1.1.1).

Bacterial gene expression depends on specific signals that are not universal between species, thus providing another molecular barrier. Expression signals can be provided either by the transformed DNA itself in the case that bacterial promoter sequences were co-transferred with the antibiotic resistance gene to the GM plant, or by a plant promoter that has promoter activity in bacteria.

(vi) the expressed resistance trait provides the bacterium with increased relative or absolute fitness.

Antibiotic resistance traits may impose additional metabolic cost for the bacterial cell but can also be of no cost. The traits increase the competitiveness of the bacterium particularly under conditions in which the corresponding antibiotics are present/used.

(vii) transfer of the antibiotic resistance gene from the initial receiving bacterium to other bacteria including human pathogenic bacteria (Chapters 2.1.2 and 2.1.3).

Once established in the recipient bacterium, the antibiotic resistance gene has the ability to spread to other bacteria. Mechanisms for this transfer are: (1) transformation; (2) transduction; and (3) conjugative transfer through plasmids and conjugative transposons. The gene flow through all these mechanisms is improved by the activity of plasmids, transposons and integrons. Multiple resistance determinants can accumulate on these mobile elements and upon transmission contribute to multidrug resistance. In general, gene transfer rates decline in a consistent manner as a function of genetic distance between bacteria. Horizontal gene transfer between distantly-related bacteria can occur through conjugation by broad-host-range plasmids and conjugative transposons.

Horizontal gene transfer can only occur where the donor and recipient bacteria share a common environment at the same time. Environments that allow frequent, multiple interactions between the donor and recipient bacteria are particularly favourable for horizontal gene transfer; *e.g.* aquatic environments, biofilms and the the mammalian gastro-intestinal system. There is increasing recognition that antibiotic consumption (human, animal and use in horticulture) provides a major selective pressure for the dissemination of antibiotic-resistant genes among bacteria. Besides direct selection by antibiotics, there could be co-selection of antibiotic resistance genes by other factors such as the presence of heavy metals, quaternary ammonium compounds, stress (DNA-damaging agents) and virulence.

4.4. Evaluation of the potential consequences of the gene transfer from GM plants to bacteria, if it occurs (hazard characterisation)

If the transfer of the antibiotic resistance marker gene from the GM plant to a bacterium would occur it could be transferred to related bacteria by conjugation, transformation and

transduction and, in the case the gene could get integrated in a plasmid or a transposable element, it could further be transferred to unrelated bacteria by conjugation, with transfer rates significantly higher than for the original transfer from the GM plant. Assuming that the transfer from the GM plant to a bacterium could occur in soil, the digestive tract or in food/feed, there would presumably be a sufficiently high density in the bacterial community to allow gene transfer among bacteria.

Aminoglycosides are used in practice in animals in the EU. If bacteria carrying the resistance marker gene *npt*II were present and retained in the animal husbandry system, either through feed and/or in the digestive tract of animals, or in animal effluents on or in the soil, selective pressure might exist and favour the dissemination of the resistance maker gene.

Prevalence data of aminoglycoside-resistant commensal and pathogenic bacteria have demonstrated that aminoglycoside-resistant bacteria are present in the food chain. Colonisation of animals by antibiotic-resistant pathogens has already been the source of infection in humans (EFSA, 2008a).

4.5. Evaluation of the overall risk posed by the transfer of antibiotic resistance marker genes from GM plants to the bacteria in the environment and the feed / food chain

The risk to human health or the environment posed by horizontal transfer of antibiotic resistance marker genes from GM plants is assessed on the basis of:

- the likelihood of horizontal gene transfer between GM plants containing ARMs and the bacteria present in their environment, and the human and animal alimentary tract; and the putative contribution of transfer from GM plant to bacteria to the existing pool of antibiotic resistance genes;
- the medical and veterinary importance of the antibiotic(s) against which such resistance is expressed.

Each step in the potential transformation process of DNA from plant cells to bacteria influences the likelihood of the transformation process, and all steps are needed for transformation to take place.

Transfer of chromosomally located antibiotic resistance genes by transformation has only been reported between closely related bacteria or, in the case of interspecies transfer, when homologous DNA was present in the recipient bacteria. Absence of homologous sequences were identified as major but not absolute barriers to horizontal gene transfer by transformation.

Compared to the transformation process between bacteria, additional barriers require to be overcome in the case of transformation of bacteria with plant-derived DNA. In particular, the absence of bacterial sequences allowing stabilisation of the transformed DNA in the bacterial cells and the lack of bacterial expression signals (promoters and terminators) limit the likelihood of the process. As the genes under consideration in this Opinion are of bacterial origin, there is a higher probability of recombination and stabilisation. If the functional antibiotic resistance gene is already present in the recipient bacterium, homologous recombination will not alter current resistance levels.

Contribution of transfer from GM plants to bacteria in the food and feed production chain

The physical processing steps of food and feed negatively affect the stability of naked DNA in most but not all cases. As a consequence these processes reduce the probability of transformation.

Transfer of antibiotic resistance genes between unrelated bacteria in the food and feed related environment has only been reported to occur by the process of conjugation. The food and feed processing and the food and feed environment have been reported to have a stimulating effect on the conjugative process.

In most but not in all cases reported, food and feed processing has been considered to have a negative effect on the transformation process.

Taken into account all the limitations of all current methodologies of detection, it can be assumed that there is, at most, a low probability of transfer of antibiotic resistance genes from GM plants to bacteria in the food and feed processing environment.

Contribution of transfer from GM plants to bacteria in the human and animal digestive tract

The human and animal digestive tract has a destructive effect on the stability of DNA. In the mouth and oesophagus DNA could survive for a sufficient time to allow uptake of DNA in the oral bacteria. No stabilization of this DNA in the recipient bacterium has ever been recorded. In the lower intestine of animals and humans no transformable genes were detected.

In the gastrointestinal tract, inhibitory effects on bacterial competence have been reported for saliva, rumen fluid and colon contents.

Transfer of antibiotic resistance genes to microbiota present in the human and animal digestive tract is only reported by the process of conjugation.

Taken into account all the limitations of all current methodologies of detection, it can be assumed that there is, at most, a low probability of transfer of antibiotic resistance genes from GM plants to bacteria in the gastrointestinal tract.

Contribution of transfer from GM plants to bacteria in the wider environment

Transfer of antibiotic resistance genes in the environment has not been demonstrated. In any event such transfer would be difficult to distinguish and quantify because of the natural occurrence of antibiotic resistance genes in such environments.

Recent metagenomic analyses of total bacterial populations (including non-culturable bacteria) have demonstrated that resistance determinants of kanamycin, neomycin and streptomycin are present in all environments investigated. The *npt*II and *aad*A antibiotic resistance genes and their phenotypes have a wide distribution, albeit at different frequencies, in different species, isolates and different environments, in naturally occurring bacteria.

Due to the use of antibiotics in human medicine and animal production worldwide and the consequent selection pressure, the prevalence of a great diversity of antibiotic resistance genes in diverse groups of bacteria has increased. Together with this, genetic structures such as plasmids, conjugative transposons, integrons and transposons, in addition to the process of conjugation, have been identified as extremely effective in retaining, accumulating and dispersing antibiotic resistance genes among bacterial populations.

Taken into account all the limitations of all current methodologies of detection, it can be assumed that there is, at most, a low probability of transfer of antibiotic resistance genes from

GM plants to bacteria in the wider environment. In arriving at such an observation, consideration has been given to the limits of the sensitivity of the methods used to detect such transfer in natural ecosystems, in view of the extent of exposure of the natural bacterial population to antibiotic-resistance marker genes introduced by the cultivation of GM plants when introduced on a global scale. Such detection is further compromised by the fact that bacterial genes derived from GM plants are indistinguishable from those already present in the bacterial population.

If this transfer would occur, it would likely take place at an extremely low frequency. Considering the presence of a great variety of genetic resistance determinants in the natural microbial reservoir, this low frequency transfer would have a limited impact.

Clinical usage and importance: observations

Most antimicrobials used for the treatment of animals belong to classes that are also used in human medicine. In a list of antimicrobial classes, compiled by WHO in 2007, and giving examples of substances used for the treatment of infections in humans and/or animals, the relevant aminoglycosides are described as being used in human medicine. Neomycin may be used for pre-operative preparation of the bowel as adjunctive therapy, and for topical application. Streptomycin was listed as a 'Critically Important Antimicrobial' for human health, and kanamycin, neomycin and spectinomycin as 'Highly Important Antimicrobials' (WHO, 2007).

The extent and mode of usage of these antibiotics in human and veterinary medicine vary from country to country. Quantification of such usage, while recorded in some countries, is difficult to interpret in the context of this Opinion. Where low quantities of a particular antibiotic are reported, one has to consider that this may be because the limited therapeutic use of such an antibiotic is due to its reserved status regarding use for specific and serious conditions. This is not to imply that bacterial resistance to antibiotics that are not listed as important either for medical or veterinary use is of no relevance to the subject of this Opinion. Given the limitations of current methodologies, it would not be prudent to regard resistance to any antibiotic as being of little or no relevance.

CONCLUSIONS

- The transfer of antibiotic resistance marker genes from GM plants to bacteria has not been shown to occur either in natural conditions or in the laboratory in the absence of sequence identity in the recipient bacterial cell. Sequence identity is necessary to allow homologous recombination between the transformed DNA in the plant and bacterial DNA.
- DNA transfer from GM plants to bacteria, if occurring, is considered to be of low frequency compared with gene transfer between bacteria.
- Recent metagenomic analyses of total bacterial populations (including non-cultivable bacteria) have demonstrated that resistance determinants of kanamycin, neomycin and streptomycin are present in all environments investigated. Such resistance genes may be selected from this environmental reservoir and disseminated among bacteria.
- The antibiotic resistance marker genes, aph(3')-IIa (*npt*II) and ant(3'')-Ia (*aad*A), in GM plants are of bacterial origin. These antibiotic resistance genes occur at different frequencies in different species, isolates and different environments, in naturally occurring bacteria. The spatio-temporal relationship between the prevalence of antibiotic resistance and selection pressure is not fully understood.
- The presence of antibiotics and antibiotic usage in different environments are key factors in driving the selection and dissemination of antibiotic resistance genes.
- Kanamycin and neomycin are both categorized by the WHO Expert Group on Critically Important Antimicrobials for Human Health as 'Highly Important Antimicrobial'. Kanamycin is used as a second-line drug for the treatment of infections with multiple drug-resistant tuberculosis (MTB). The increasing occurrence worldwide of "extensively drug-resistant" (XTB) isolates of MTB with resistance to second-line antibiotics such as kanamycin is a cause for global concern. The *npt*II gene has not been implicated in such resistance. The above WHO group has also categorised streptomycin as a 'Critically Important Antimicrobial', and spectinomycin as a 'Highly Important Antimicrobial'.
- There are limitations related among others to sampling, detection, challenges in estimating exposure levels and the inability to assign transferable resistance genes to a defined source. The importance of taking these and other uncertainties described in this Opinion into account requires to be stressed.
- Notwithstanding these uncertainties, the current state of knowledge indicates that adverse effects on human health and the environment resulting from the transfer of these two antibiotic resistance genes from GM plants to bacteria, associated with use of GM plants, are unlikely.

DOCUMENTATION PROVIDED TO EFSA

1. Letter from DG SANCO and DG ENV, dated 14 May 2008, concerning the mandate for the use of antibiotic resistance marker (ARM) genes used as marker genes in genetically modified plants (ref. SANCO/E1/SP/pm (2008) D/510274).
2. Enclosure 1. May 2008. Submitted by European Commission.

   Letter from Greenpeace to the Commissioner for Health and Food Safety, dated 13 February 2008, concerning the authorisation of GM BASF potato EH92-527-1 / Agriculture Council 18 February 2008.
3. Enclosure 2. May 2008. Submitted by European Commission.

   Letter from the Danish Minister for Food, Agriculture and Fisheries and Minister of Environment to Commissioner Vassiliou and Commissioner Dimas, dated 14 March 2008.
4. Enclosure 3. May 2008. Submitted by European Commission.

   Email message from Greenpeace to Commissioner Dimas, dated 13 September 2007, concerning the Institute Pasteur study on antibiotic resistance and GM plants.

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APPENDICES

Appendix A

EVALUATION OF ANTIBIOTIC RESISTANCE GENE WITH MARKER FUNCTION IN PLANTS: *aph(3´)*-IIa

1. Introduction

The *aph*(3´)-IIa gene is often referred to as *npt*II gene or kanamycin resistance gene because of the use of kanamycin to select for recombinant cells. Its phosphorylation of neomycin gives the gene its acronym – standing for neomycin phosphotransferase. It has been used in the genetic modification of organisms as diverse as bacteria, yeasts, plants and animals. Plant cells that produce the APH(3')-IIa enzyme selectively survive exposure to kanamycin by rendering the antibiotic nontoxic (De Block *et al.*, 1984; Horsch *et al.*, 1984).

The safety of *aph*(3´)-IIa has been discussed, *e.g.* by Flavell *et al*. (1992), Nap *et al*. (1992), Fuchs *et al*. (1993a, b), Redenbaugh *et al.* (1994) and Goldstein *et al.* (2005). The assurance of safety of APH(3')-IIa protein is based on the lack of structural similarity to known toxins, acute toxicity study on mice, low concentration of the protein in plant tissues, and rapid digestibility in simulated digestive fluids.

2. Origin of *aph*(3´)-IIa

The gene *aph*(3´)-IIa (also called *npt*II, *neo*, *kan*, *aphA*-2) originates from the transposon Tn*5* (Garfinkel *et al.,* 1981; Beck *et al*., 1982; Mazodier *et al*., 1985). Transposon Tn*5* was originally isolated from *Klebsiella pneumoniae*, but was subsequently found in a number of bacteria including *Escherichia coli*. The *aph(3´)*-IIa gene encodes the APH(3')-II protein (ATP:kanamycin 3'-O-phosphotransferase; aminoglycoside-3'-phosphotransferase) [EC 2.7.1.95; Chemical Abstracts Registry Number (CAS): 62213-36-9; UniProtKB/Swiss-Prot P00552 (KKA2_KLEPN)]. The protein used for genetic modification of plants is presently designated APH(3')-IIa (type II aminoglycoside-3'-phosphotransferase; type II kanamycin kinase) to distinguish it from another protein (with 51.7% amino acid sequence identity) isolated later from *Pseudomonas aeruginosa* and designated APH(3')-IIb (Hächler *et al*., 1996). The 3' refers to the particular hydroxyl group in the aminoglycoside molecule that is phosphorylated by the enzyme; numerous other bacterial aminoglycoside-phosphotransferases catalyze the phosphorylation of other hydroxyl groups in the aminoglycoside molecules. Type II refers to the classification which is based on the aminoglycoside resistance pattern conferred to the bacterium containing a particular APH(3') enzyme (there are seven different classes: I-VII) (Wright and Thompson, 1999).

3. Food and feed safety assessment of APH(3')-IIa: Toxic, allergenic or other harmful effects on human or animal health

3.1. Toxicological assessment of APH(3')-IIa protein

3.1.1. Homology to known toxins: bioinformatics analysis

Amino acid sequence similarities of APH(3')-IIa to all protein sequences in the publicly available databases have been evaluated using bioinformatics tools. No toxicity concerns have been revealed by these analyses.

3.1.2. Exposure to APH(3')-IIa protein

The extent of normal exposure to protein variants is not well known but is easily in tens of thousands, and probably in the order of 100,000. A eukaryotic cell contains 5.000 to 10.000 different polypeptides that must be degraded to produce the amino acids required for growth. When this number is multiplied by a factor to account for tissue-specific differences and by the number of different species that are eaten the number of proteins in the diet becomes very large. Genetic polymorphism also contributes to the total dietary protein array.

Levels of APH(3')-IIa in GM plants

The APH(3')-IIa levels in various parts of the GM plants varied from levels below the limit of detection (maize grain) to less than 34 µg/g (0.0034%) tissue fresh weight (cotton leaf). The expression tended to be higher with the 35S promoter (used in maize and in several transgenic cotton lines) than with the *nos* promoter, which is known to be a "weaker" promoter (used in potato). Examples of the levels of APH(3')-IIa compared with total protein are 0.0006% in potato tubers and 0.0028% in cottonseed (MON 1445). By assuming that cottonseed protein is approximately 22% of seed weight, the maximum amount of APH(3')-IIa in 1 gram of unprocessed cottonseed would be 6.2 µg. Redenbaugh *et al*. (1994) gave an example of BXN™ cottonseed, in which the concentration of APH(3')-IIa was less than 0.003% of the total seed protein. Similarly, the levels of APH(3')-IIa in High Stearate rapeseed whole seed and leaves were less than 0.0008% of the protein.

Stability of APH(3')-IIa during processing

Food that potentially carries the highest risk is that which is consumed fresh. None of the plant parts considered in this opinion is intended for consumption without any processing. Maize forage and potato pulp, both intended for feed use, contained *ca*. 0.2 µg and 0.05 µg of APH(3')-IIa per g tissue fresh weight, respectively. No APH(3')-IIa protein could be detected from fibre fractions obtained from cotton or starch fraction obtained from potato. The activity of APH(3')-IIa was lost upon processing/toasting used to remove cottonseed oil and less than 4% of the total APH(3')-IIa protein was found to be present in the processed raw cottonseed meal. The low amount of protein present in the meal was obviously in non-functional, denatured form due to the high temperature during processing. Redenbaugh *et al*. (1994) reported that while the level of APH(3')-IIa was less than 0.003% of the protein in unprocessed BXN™ cottonseed, the level in processed cottonseed meal was less than 0.0014%. Similarly, the levels of APH(3')-IIa in High Stearate rapeseed whole seed and leaves was less than 0.0008% of the protein and less than 0.00008% of protein in the processed meal. These examples demonstrate that processing further decreases the amount and activity of APH(3')-IIa, being non-detectable in highly purified products such as starch or fibre. Seeds are often protected by a resilient seed coat; the DNA remains intact through the gut but may not be available for gene transfer.

Exposure to food/feed

Dietary intake of APH(3')-IIa protein through GM food (whether processed or not) is low or non-existing. Upper bound estimates of human daily maize grain consumption were used to calculate the margin-of-exposure ("safety margin") for APH(3')-IIa (MOE is the ratio of the no-effect-level, derived from toxicology tests, to the estimated human daily-dietary-exposure). An MOE ≥ 100 is generally regarded as being adequate to protect human health. The NOEL (for mice) is 5000 mg/kg body weight (see below). For maize grain the calculated MOE was $\geq 1.53 \times 10^8$, indicating that the risk to human health is very low. The primary cotton products used for human food, *i.e.* cottonseed oil and processed cotton linters contain no detectable protein. The potato starch contains no APH(3')-IIa protein.

For dietary consumption of APH(3')-IIa protein from maize forage by dairy cows the calculated MOE was $\geq 5.8 \times 10^5$, again indicating very low exposure. Given the presence of 55 ng APH(3')-IIa/g fresh weight of the potato-derived pulp, and a daily ration of a maximum of 20 kg fresh pulp per cattle, the daily consumption of APH(3')-IIa protein per kg of body weight derived from the pulp would be 0.0022 mg. This yields an MOE of $>2.2 \times 10^6$ for APH(3')-IIa in pulp.

Digestion in simulated digestive fluids

Most proteins in food are rapidly degraded upon consumption and exposure to the proteases and acidic conditions of the mammalian digestive tract. An *in vitro* digestion system is widely used to study the digestibility of proteins. The purified APH(3')-IIa protein is rapidly degraded in simulated gastric (pepsin, pH 1.0) and intestinal (pancreatine, pH 7.5) fluids (Fuchs *et al.*, 1993a, b). The protein is readily degraded in both fluids, with half lives of <10 seconds and between 2 and 5 minutes in gastric and intestinal fluids, respectively. The disappearance of the protein was consistent with the loss of enzymatic activity of APH(3')-IIa. The degradability of APH(3')-IIa protein has also been tested in anaerobic conditions in ruminal fluid originated from fistulated sheep (EFSA, 2006c). Quantities of protein used in the test were within the range of expected practical levels (0.1 and 1.0 ng/ml) but also 100 ng/ml was tested. Even at the highest concentrations the protein degraded rapidly, most of it in a few minutes.

The conclusion is that APH(3')-IIa protein is rapidly degraded in simulated digestive fluids. This suggests that in the stomach and small intestine most, if not all, APH(3')-IIa protein will be inactivated or degraded by the acidic environment and digestive enzymes. Under simulated abnormal conditions in neutralised gastric fluid (which may exist in patients treated with drugs that reduce stomach acidity) the enzyme may remain active. While poor digestibility of a protein might be an indication of allergenic potential, these studies did not raise such concerns.

3.1.3. Acute oral toxicity study on mice with APH(3')-IIa protein

The rationale for conducting acute oral toxicity study is that proteins that are toxic to animals produce toxic effects following acute exposure. Acute toxicity of APH(3')-IIa has been tested via gavage administration of three doses of purified protein (100, 1000 and 5000 mg/kg of body weight) to 10 male and 10 female mice (Fuchs *et al*., 1993 a, b). The highest dose corresponded to the Maximum Hazard Dose stated in the guidelines from the US Environmental Protection Agency for biochemical pesticides and would be equivalent to an average human consuming, in one day, of more than one million tomato fruit or potato tuber expressing the level of APH(3')-IIa protein that had been reported for those GM crops. There

was no mortality, no adverse reactions and no differences attributed to treatment in body weight or gain or food consumption compared with untreated mice. No abnormal changes were observed in the tissues of mice necropsied approximately eight days after dosing. It was concluded that the LD_{50} for APH(3')-IIa is >5000 mg/kg body weight and the NOEL is 5000 mg/kg body weight. This dosage correlates to at least a million fold safety factor relative to the average daily consumption of GM potato or GM tomato containing APH(3')-IIa. This result confirms that the APH(3')-IIa protein has no acute toxicity.

3.1.4. Subchronic toxicity study on rats with unprocessed potato containing APH(3')-IIa

A 90-day rat study was conducted with a diet containing 5% of the unprocessed (raw, freeze-dried) GM potato. The study did not reveal any effects that would raise concerns about the safety of the potato (EFSA, 2006c).

3.1.5. Human gene therapy

The *npt*II gene has been used as a selectable marker gene in a number of clinical studies involving human gene therapy (*e.g*. Brenner *et al*., 1993). The safety of APH(3')-IIa has been thus demonstrated directly in humans with no clinically observable side effects (Rosenberg, 1991; reviewed by Redenbaugh *et al.*, 1994).

3.1.6. Nutritional studies on animals with feed containing APH(3')-IIa

Nutritional studies have been conducted on feed containing APH(3')-IIa protein. While these cannot be considered as toxicity studies, they can provide further assurance of the safety of the APH(3')-IIa protein. A feeding study on broilers was performed with GM maize. No substance-related findings were observed. The NOAEL (no-observed-adverse-effect-level) for male and female Wistar rats was higher than 3731 or 4374 mg/kg bw/day, respectively. An 8-week feeding study on heifers was performed with the pulp of GM potato (EFSA, 2006c). No significant differences in feed intake or weight gain were observed between animals fed on pulp derived from GM or non-GM potato. No effects were observed on animal health and intestinal functions.

3.2. Allergenicity

The following aspects have been taken into consideration in terms of potential allergenicity of APH(3')-IIa protein: 1) The in vitro digestibility study on the APH(3')-IIa protein (see above) did not raise any concerns; 2) Glycosylation and subsequent increase in the antigenic capacity of APH(3')-IIa would not occur because APH(3')-IIa does not contain the necessary sequence information for transport to the subcellular locations at which glycosylation reactions take place (Redenbaugh *et al*., 1994); 3) Amino acid sequence comparison of APH(3')-IIa to all protein sequences in the publicly available genetic databases has not revealed any significant matches to known allergens; 4) No allergenicity from APH(3')-IIa was found in human gene therapy studies. On this basis it has been concluded that APH(3')-IIa raises no specific concerns.

3.3. Other harmful effects on human or animal health

A major concern is the potential of APH(3')-IIa to interfere with antibiotic therapy of humans and animals. To assess the possibility, it is important to consider the catalytic activity and

substrate specificity of APH(3')-IIa protein as well as the chances for the *aph(3')*-IIa gene to move from the GM crop to human/animal pathogens.

3.3.1. Catalytic activity

Aminoglycoside antibiotics, some of which are substrates for APH(3')-IIa protein, bind to 16S ribosomal RNA of the 30S ribosomal subunit, decreasing the dissociation rate of aminoacyl-tRNA and causing misreading of messenger RNA (Yoshizawa *et al*., 1998; Carter *et al*., 2000; Lynch *et al*., 2003). This leads to the interference of protein synthesis, resulting in the synthesis of non-functional, misfolded proteins. The complex three-dimensional folds of RNA serve as specific targets for the antibiotics, but at the same time the high conservation of the target sites across species may be problematic in terms of toxicity (the aminoglycosides are toxic not only to pathogenic bacteria but also to beneficial bacteria and to human cells); however, the bacterial ribosome is sufficiently different from the eukaryotic cytoplasmic ribosome to have made protein synthesis a target for the design of antibiotics. This is not the case for mitochondrial or chloroplast ribosomes but there are permeability barriers that protect these structures. As the aminoglycosides are also toxic to plant cells the system involving the modification of an aminoglycoside, *i.e*. kanamycin, to a non-toxic metabolite has been exploited in the generation of GM plants.

The APH(3')-IIa protein (type II aminoglycoside-3'-phosphotransferase, neomycin phosphotransferase) encoded by the *aph(3')*-IIa gene catalyzes the phosphorylation of the 3'-hydroxyl group of the aminohexose moiety of certain aminoglycoside antibiotics (Bryan, 1984). The modified aminoglycoside can no longer bind to the 30S ribosomal subunit and can no longer cause misreading of mRNA and inhibition of protein synthesis. Since the phosphorylation is ATP-dependent, ATP has to be present in sufficient amounts for the catalytic reaction to take place.

3.3.2. Substrate specificity

Even though very similar in structure, aminoglycosides are recognised differently by the different aminoglycoside modifying enzymes, even by different aminoglycoside phosphotransferases. Thus the APH(3')-IIa protein is able to phosphorylate only a subset of aminoglycosides (Table A1). Kanamycin and neomycin are very good substrates for APH(3')-IIa, whereas, for example, G418 and amikacin are inefficient substrates (Perlin and Lerner, 1986; Siregar *et al*., 1994). What determines the substrate specificity is of particular interest. It is believed that electrostatic interactions (ion pairing and hydrogen bonding) are significant for both substrate binding and catalysis among aminoglycoside-modifying enzymes (Azucena and Mobashery, 2001). Nurizzo *et al*. (2003) determined the high resolution (2.1 Å) crystal structure of APH(3')-IIa in complex with kanamycin in order to gain a better understanding of the molecular basis for aminoglycoside recognition and inactivation. The pattern of secondary structure found by Stoldt *et al*. (2004) by using NMR (nuclear magnetic resonance) spectroscopy was in agreement with the crystallographic structure. Nurizzo *et al*. (2003) suggested that the differences in aminoglycoside substrate specificity and resistance profile of different phosphotransferases arise in large part from the sequence and conformational variability of the long acidic α4-α5 loop which forms interactions with the B- and C-rings of kanamycin.

There is often confusion about the substrate specificity of APH(3')-IIa towards gentamycin. The clinically used gentamicin is a mixture of gentamicins C_1, C_{1a} and C_2 (Table A1).

APH(3') enzymes modify kanamycin and related compounds at the 3'-hydroxyl group. Thus compounds such as the gentamicin C_1, C_{1a} and C_2 and tobramycin which lack this functionality, are not substrates for these APH(3') enzymes, However, gentamicin A_2 and gentamicin B which possess the 3'-hydroxyl group (Wright and Thompson, 1999) are substrates for these enzymes.

Table A1. Substrate specificity of APH(3´)-IIa encoded by *aph(3´)*-IIa (for the structures of the aminoglycosides see, *e.g.* Azucena and Mobashery, 2001)

Aminoglycoside tested	Modified by APH(3')-II	MIC (mg/L)	K_m (µM)[d]	Reference
Kanamycin (A)[a]	yes	*Escherichia coli* strain without *aph(3´)*-IIa: 1.56; same *E. coli* strain with *aph(3´)*-IIa: 1600 *Escherichia coli* without aph(3´)-IIa: 1.56 Same *E. coli* with *aph(3´)*-IIa: 800	3; 10	Nap *et al*., 1992 and references therein; Siregar *et al*., 1994; Nurizzo *et al*., 2003; Perlin and Lerner, 1986
Neomycin (B)	yes	*Escherichia coli* strain without *aph(3´)*-IIa: ≤ 0.78; same *E. coli* strain with *aph(3´)*-IIa: >800	6	Nap *et al*., 1992 and references therein; Siregar *et al*., 1994; Nurizzo *et al*., 2003
Ribostamycin	yes		10	Perlin and Lerner, 1986
Butirosin	yes		25.5	Azucena and Mobashery, 2001; Perlin and Lerner, 1986
Lividomycin	yes			Azucena and Mobashery, 2001
Gentamicin A	yes			
Gentamicin B	yes	*Escherichia coli* strain without *aph(3´)*-IIa: ≤0.78; same *E. coli* strain with *aph(3´)*-IIa: 100		Nurizzo *et al*., 2003
Geneticin (G418)	yes		15	Siregar *et al*., 1994
Neamine	yes		1	Siregar *et al*., 1994
Amikacin	yes[b]	EUCAST sensitivity breakpoint: 8; *Escherichia coli* strain without *aph(3´)*-IIa: ≤0.78; same *E. coli* strain with *aph(3´)*-IIa: 6.25 *E. coli* without *aph(3´)*-IIa: 0.78; Same *E. coli* with *aph(3´)*-IIa: 3.12	53; 720	Nurizzo *et al*., 2003; Siregar *et al*., 1994; Perlin and Lerner, 1986
Gentamicin[c]	no	*Escherichia coli* without aph(3´)-IIa: 1.56 Same *E. coli* with *aph(3´)*-IIa: 0.78 (gentamicin from Schering, not specified)		Nap *et al*., 1992 and references therein; Perlin and Lerner, 1986
Netilmicin	no			Nap *et al*., 1992 and references therein

[a]The **kanamycins** are a family of three antibiotics, kanamycin A, B and C. The disulfide salt of kanamycin A is the most widely used of these three antibiotics. It is assumed that whenever no detailed specification is given, the studies refer to kanamycin A or a mixture where the other kanamycin forms are present as minor components. The crystal structure, conformation and absolute configuration of kanamycin A were determined by Puius *et al*. (2006). Kanamycin A exists in a long extended conformation with all three rings in the chair conformation. The conformation of amikacin is very similar (conformation of the A/B rings is essentially unchanged) to that of kanamycin A. However, the B/C ring junctions are significantly different due to the change of the NH_2 group at the N1 position of kanamycin by the long hydroxyaminobutyryl side chain in amikacin.

[b]Activity of the purified enzyme for **amikacin** is detectable *in vitro* (Siregar *et al*., 1994), but the presence of the enzyme in bacterium does not confer resistance to amikacin due to the slow catalysis (Nap *et al*., 1992 and references therein). This is an example where one cannot necessarily correlate quantitatively the level of activity of an aminoglycoside-modifying enzyme, as determined by an *in vitro* assay, with the level of resistance for which it is responsible.

[c]The clinically used **gentamicin** is a mixture of gentamicins C_1, C_{1a} and C_2 having different patterns of methylation (http://www.inchem.org/documents/pims/pharm/gentamic.htm; last accessed 22.9.2008; Yoshizawa *et al*., 1998). APH(3') enzymes modify kanamycin and related compounds at the 3'-hydroxyl group. Thus compounds such as the gentamicin Cs and tobramycin which lack this functionality, are not substrates for these enzymes as opposed to gentamicin A_2 (first pseudotrisaccharide intermediate in the biosynthesis pathway for the gentamicin complex in

Micromonospora echinospora; Park *et al*., 2008) and gentamicin B which possess the 3'-hydroxyl group (Wright and Thompson, 1999). An exception of the rule is lividomycin which is phosphorylated at the 5''-hydroxyl group by several APH(3')s.

[d]Resistance is almost invariably more effectively specified to a substrate with a lower K_m than to a substrate with a higher K_m. It is probable that the K_m is the more critical factor than the rate of enzymatic activity in order of the reaction to be effective at clinically significant concentrations of the aminoglycoside (Bryan, 1984).

3.3.3. Compatibility of antibiotic therapy with the presence of low levels of APH(3')-IIa protein in food and feed

A question has been raised whether the consumption of food containing APH(3')-IIa enzyme could interfere with the oral therapeutic usage of aminoglycoside antibiotics. To decide on this, it is necessary to consider the requirements for the catalytic reaction to take place (Appendix A – 3.3.1.) and the degradability of the APH(3')-IIa protein in the gastrointestinal tract (Appendix A – 3.1.2.) besides the substrate specificity of APH(3')-IIa (Appendix A – 3.3.2.) and the mode of use of the aminoglycosides (Chapter 3).

That the oral use of the antibiotics would not be compromised by the presence of APH(3')-IIa in the diet is derived from several considerations: 1) The APH(3')-IIa enzyme is rapidly degraded in the gastrointestinal tract; 2) The APH(3')-IIa enzyme, as proteins in general, are poorly absorbed by the digestive system; 3) The enzymatic catalysis requires ATP, which is present in the digestive system at extremely low levels because it is unstable at low pH; 4) Only very small proportion of kanamycin or neomycin are administered orally or used for gastrointestinal tract.

It has been calculated that compromising the efficiency of oral neomycin therapy due to ingestion of GM foods containing APH(3')-IIa together with fruits and vegetables rich in ATP would also be extremely unlikely (Redenbaugh *et al.*, 1993, 1994). Using GM tomato expressing the APH(3')-IIa 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 1.5%. The number is based on the following assumptions: 1) 95th percentile consumption, at a single serving, of fruits or vegetables high in ATP; 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 (a highly unlikely situation); 4) administration of neomycin simultaneously with consumption of a GM food containing APH(3')-IIa and other fruits or vegetables rich in ATP; 5) presence of intact, functional APH(3')-IIa 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')-IIa present in food.

Potential inactivation of neomycin in feed during storage due to activity of APH(3')-IIa has been assessed in cottonseed meal and rapeseed meal (Redenbaugh *et al*., 1994). The stability of antimicrobial activity was determined by bioassay. After 8 weeks of storage at 37ºC, the meals showed no significant decline in neomycin levels that could be attributed to the presence of the APH(3')-IIa enzyme. After the storage period no APH(3')-IIa activity was observed. Thus there is no discernible risk that meal from GM cottonseed or rapeseed containing the APH(3')-IIa would compromise the efficacy of neomycin in feed.

3.3.4. Exposure of humans/animals to bacteria that possibly acquire *aph*(3')-IIa from plant

The amount of *aph(3')*-IIa DNA is very low compared with the total ingested DNA from food and the *aph(3')*-IIa genes do not contain any special features that would render them more resistant to digestion in the gastrointestinal tract than any other DNA. In the following, examples of the calculations are given.

DNA is consumed daily in most of the food that people eat (*e.g.* fruit, vegetables, *etc*.). The daily dietary intake of a 5 kb piece of DNA inserted in the genome of MON 863 maize has been estimated as 0.049 mg/day per capita, based on Austrian food balance sheet (Jonas *et al*., 2001). This upper estimate value assumed that all the ingested maize would contain a 5 kb insert. For maize lines derived from MON 863 transformation event, the *aph(3')*-IIa gene represents less than 1 x 10^{-4}% (2 kb/2x10^{6} kb) of the plant nuclear genome. The expected exposure of intact *aph*(3')-IIa gene released by MON 863 event to bacteria in the human gut is therefore estimated to be extremely low. Redenbaugh *et al*. (1993) estimated that the mean consumption rate of the *aph*(3')-IIa gene in fresh GM tomatoes would be 3.3 x 10^{-4} ng/day. The amount of eukaryotic DNA that is shed into the lumen of the digestive tract over a 24-h period has been calculated to be 5 mg in the stomach, 200 to 500 mg in the small intestine, and 20 to 50 mg in the colon. Thus the amount of *aph*(3')-IIa DNA is several orders of magnitude lower than the quantity and variety of DNA normally present in the digestive tract.

The likelihood of gene transfer from plant to a human pathogen via any known or putative routes is extremely low (Chapter 2.1.). Furthermore, humans continually ingest kanamycin-resistant microorganisms. Fresh salad and vegetables are major sources and each human is estimated to ingest 1.2x10^{6} kanamycin-resistant microorganisms daily (Flavell *et al*., 1992). Approximately 10^{14} bacteria are present in the digestive tract. Nap *et al*. (1992) presented a theoretical calculation suggesting that consumption of 250 g GM tomatoes would generate 0.0024 transformants, which corresponds to an increase of 2.4 x 10^{-15}% of the total intestinal microbiota, estimated as less than the natural supplement through spontaneous mutations. To reach that conclusion it was assumed that: all transformable bacteria are *E. coli*; the transformation efficiency of plasmid DNA is 10^{-6}; linear DNA is transferred at 0.1% the frequency of circular plasmid DNA; 10 copies of *aph*(3')-IIa are present per tomato genome; there is no breakdown of DNA in the intestinal tract. Furthermore, recombination and subsequent selection should occur to make the gene functional and effective. Therefore the actual contribution of the *aph*(3')-IIa gene would be considerably smaller. Kharazmi *et al*. (2003) estimated the likelihood of transformation of *Bacillus subtilis* with *aph*(3')-IIa present within transgenic potatoes. This estimation was based upon the transformation frequency that these authors had observed after incubating *B. subtilis* that contained a partially deleted *aph*(3')-IIa gene with plasmid and linear DNA molecules that contained homologous fragments of the same gene. It was thus estimated that the world population would have to consume potatoes for 15 days in order to be exposed to one transformant of *B. subtilis* in food by means of homologous recombination. This estimated frequency will likely be lower in the absence of homologous sequences in the recipient microorganisms (Kharazmi *et al*., 2003).

It has been calculated that under worst case and extremely rare conditions, the potential increase in the number of gut bacteria that could become resistant to kanamycin is 0.00000000000026% in a human that has consumed the 90th percentile level of fresh tomatoes (Redenbaugh *et al.*, 1994). This was interpreted as meaning that for every 380 humans that consume the mean level of GM tomatoes, one gut bacterium susceptible to kanamycin might become resistant.

4. Environmental risk assessment

4.1. Potential effects on plant fitness

Kanamycin resistance gives the transformed cells a selective advantage in the presence of the antibiotic. Thus, kanamycin resistance can contribute to the selective advantage of the transgenic plant only when selective concentrations of the antibiotic are present in nature. Evaluation of the probability of the presence of such antibiotic levels will give insight into the potential weediness of kanamycin resistant plants. There are two ways for the emergence of such selective conditions: (1) antibiotic production in the soil; and (2) addition of the antibiotic into the soil (reviewed by Nap *et al*., 1992).

Kanamycin and neomycin are produced by soil microorganisms. Veterinary use of kanamycin and neomycin is a potential source of antibiotic addition to soil; both antibiotics are poorly resorbed in the gastrointestinal tract and an estimated 97% leaves the body unchanged in the faeces. However, the conclusion from several studies and estimates is that the amount of soil-produced kanamycin will be small and that the physico-chemical characteristics of the antibiotics and the soils will result in conditions that give kanamycin-resistant plants no selective advantage over the susceptible parent plants. It can be excluded that any soil will be able to accumulate kanamycin or neomycin in concentrations that are selective for GM plants. Kanamycin resistance will therefore not contribute to enhanced weediness of a plant. Available data also show that the APH(3')-IIa enzyme does not interfere with the basic functions required for normal plant growth and development.

4.2. Potential for gene transfer

4.2.1. Plant to plant gene transfer

If the *aph*(3')-IIa gene would move from a crop plant to related plants by pollination, its effects on the recipient plant would not be expected to be different from those on the donor plant. Thus the transfer of *aph*(3')-IIa to other crops or related weeds will have no significant adverse environmental effects. Wild relatives that receive the gene would only become less controllable than the parent plant in the presence of selective antibiotic. As argued above, this situation will not occur. Large plant families are already resistant to kanamycin (Nap *et al*., 1992; Mentewab and Stewart, 2005; Rommens, 2006; Burris *et al*., 2008). Therefore, kanamycin resistance should not be considered a novel characteristic for any ecosystem and will not contribute to enhanced weediness of any crop or its wild relatives.

4.2.2. Plant to bacteria gene transfer

The *aph*(3')-IIa gene is of bacterial origin, and kanamycin-resistant bacteria are detected in all soils investigated (Chapter 2.2.). Many of the genes conferring kanamycin resistance are transferred between bacteria because they reside on transposons and often on transmissible plasmids (Chapter 2.1.2.). Some calculations are shown below.

Nap *et al*. (1992) concluded that transfer of *aph*(3')-IIa from tomato plants would result in 8.7 x 10^{-5} GM bacteria per gram of soil (transformation frequency 8.7 x 10^{-12}). In the calculation, Nap *et al*. (1992) used a "worst case scenario": 1) all tomato plants including all fruits annually produced in The Netherlands, assuming 10 copies of *aph*(3')-IIa are ploughed into the first 30 cm of the area in which tomato plants are cultivated; 2) 10% of the DNA is released from the plants; 3) 10% of the released DNA is intact; 4) 10% of the soil organisms are transformable, of which 5% are competent; 5) natural transformation frequency is 0.01%.

This calculation did not take into account the fact that the *aph*(3')-IIa gene functional in plant carries regulatory sequences that will generally not work in microorganisms, and that there should be selective advantage of kanamycin resistance so that the bacterium would be able to outcompete the kanamycin-resistant organisms already present in the soil.

One gram of soil contains up to 10^9 microorganisms (D'Costa *et al*., 2007). Redenbaugh *et al*. (1994) estimated that kanamycin-resistant transformants resulting from plant DNA leaf in the fields would constitute not more than 1/10.000.000 of the existing kanamycin-resistant soil population. The most realistic estimate made was that kanamycin-resistant bacteria resulting from transformation by plant DNA would represent an addition of about one organism to the total number of kanamycin-resistant soil microorganisms present in one hectare.

5. Prevalence of *aph(3')*-IIa gene

A misconception occasionally associated with the introduction of GM food is that the only source of the resistance genes is the GM food. However, the gene originates from bacteria that are commonly encountered by humans and their domestic animals. The kanamycin resistance gene is not novel to the food supply, as the gene can be found in contaminating bacteria on or in food (Chapter 2.2.).

6. Conclusion

The safety of the *aph*(3')-IIa gene and its protein product APH(3')-IIa has been verified by a number of studies. The exposure of humans and animals to the gene and protein via food and feed is very low due to the initially low levels in plants and further losses during processing. The protein is readily digested in the gastrointestinal tract. Bioinformatic analyses indicate no concerns as regards toxicity or allergenicity. Lack of toxicity has been verified by acute oral toxicity in mice. The *aph*(3')-IIa gene has been used in human gene therapy studies with no clinical signs of toxicity. Subchronic toxicity study on rats and nutritional studies on broilers and heifers with plant material containing APH(3')-IIa provide further assurance of safety.

The substrate specificity of APH(3')-IIa and resistance profile given by *aph*(3')-IIa indicate that two highly critical antibiotics, amikacin and gentamicin C, are not affected. The *aph*(3')-IIa gene does not increase the fitness of the GM plant.

Appendix B

EVALUATION OF ANTIBIOTIC RESISTANCE GENE WITH MARKER FUNCTION IN BACTERIA USED IN THE CONSTRUCTION OF GENE CASSETTES FOR TRANSFER TO PLANTS: *ant(3'')*-Ia

1. Introduction

The *ant(3'')*-Ia [= *aad*A, Strep/Spec^R] gene is often referred to as the streptomycin resistance gene. This resistance gene is used as a marker in bacterial gene constructs that are intended to be transferred in the plant. The primary function of *ant(3'')*-Ia is to facilitate the isolation of bacterial colonies that have been transformed with recombinant plasmid DNA and not to serve as a marker to select for recombinant plant cells. However, the gene can be intentionally or unintentionally be introduced in plant cells during the process of genetic modification. The *ant(3'')*-Ia gene is a widely used gene during the process of genetic modification of all kind of (micro)organisms. The gene encodes streptomycin adenyltransferase (Davies and Smith, 1978) which modifies the 3"-hydroxyl position of streptomycin and the 9-hydroxyl position of spectinomycin (Shaw *et al*., 1993).

2. Origin of *ant(3'')*-Ia

Streptomycin resistance has been studied in clinical bacteria and in agriculture. So far, only four types of streptomycin inactivating genes are known, being aminoglycoside phosphotransferases *aph*(6) and *aph*(3") and aminoglycoside nucleotidyltransferases *ant*(6) and *ant*(3") (Shaw *et al*., 1993). The conservation of these four mechanisms in both Gram-negative and Gram-positive bacteria suggests that gene transfer has played an important role in the dissemination of streptomycin resistance genes (Sundin and Bender, 1993). The *ant*(3") or *aadA* gene originates from the plasmid R538-1 of *Escherichia coli.* The gene is commonly encountered in enteric, Gram-negative bacteria and has been cloned from several transposons (Shaw *et al*., 1993). Tomalsky and Crosa (1987) detected the *ant(3'')*-Ia (Strep/Spec^R) gene on the multiresistance transposon Tn1331 in *Klebsiella pneumonia.* Since then, a wide range of scientific publications have appeared describing the occurrence of *aadA* gene on various transposons and integron 1 amongst a wide range of bacteria from various environments, such as in soil, sludge, wastewater, seawater (Van Overbeek *et al*., 2002), irrigation water (Roe *et al*., 2003), food, human and animal digesta/feces and in human clinical specimens (*e.g.* Barlow *et al*., 2008; Nandi *et al*., 2004; Van *et al*., 2008; Srinivasan *et al*., 2007; Enne *et al*., 2008; Zhao *et al*., 2003) (see also Chapter 2.2.),

3. Safety assessment of ANT(3'')-Ia protein for human/animal health and the environment

The *ant(3'')*-Ia gene is only present in several cotton events, all derivatives of MON531 and MON1445 (Appendix C). In all these events, the *ant(3'')*-Ia gene is under control of its own bacterial promoter and terminator and is thus not expressed in the cotton plants. The lack of detectable expression of the *ant(3'')*-Ia gene was confirmed in expression studies of these events (SCP opinions MON531 and MON1445) (SCP, 1998a, b). Therefore, no ANT(3'')-Ia protein will be present in the cotton tissues of the cotton events. As a consequence, the food and feed safety of the ANT(3'')-Ia protein is not considered during the risk assessment of these cotton events.

(http://europa.eu.int/comm/food/fs/sc/scp/out18_en.html, http://europa.eu.int/comm/food/fs/sc/scp/out17_en.html).

4. Safety assessment of the *ant(3'')*-Ia gene for human/animal health and the environment

The *ant(3'')*-Ia gene, though not expressed, is present in the plant material during growth and consumption by humans and animals. The only route via which the gene can potentially pose a risk is by functional gene transfer to harmful bacteria. This can only occur in case the DNA is not degraded, is taken up by bacteria through transformation and is expressed (see also Chapter 2.1.). The persistence of plant DNA in the environment is proven to be very low, but small amounts of DNA from GM plants can persist and transform bacteria (Paget and Simonet, 1994). GM plant material intended for use in food of feed is often subject to a variety of processing and storage regimes. Food/feed processing and extraction of ingredients physically and chemically damage and degrade DNA and this limits gene transfer (Kharazmi *et al*., 2003). In case of cotton, the only products consumed by humans are refined cottonseed oil and cellulose from processed linters of cottonseed. Processed linters are essentially pure cellulose (>99%) and are subjected to heat and solvent treatment that would be expected to remove and destroy DNA and protein. Similarly, the refining process for cottonseed oil includes heat, solvent and alkali treatments that would remove and destroy DNA (*http://www.agbios.com*). The defatted seed meal remaining after oil extraction is used as animal feed, the bulk of which is fed to ruminants and a limited amount of cottonseed meal is used in the diets of pigs, poultry and fish. The physical and heat treatment used to obtain maximum oil recovery is adequate to damage any DNA present substantially (SCP, 1998a, b) (http://europa.eu.int/comm/food/fs/sc/scp/out18_en.html, http://europa.eu.int/comm/food/fs/sc/scp/out17_en.html).

Any potentially remaining DNA from the *ant(3'')*-Ia gene in food or feed products is unlikely to persist in the digestive tract (from mouth to colon). DNA is expected to be degraded rapidly to short peptides and amino acids (Ramessar *et al*., 2007) (see also Chapter 2.1.1.1.).

4.1. Exposure of humans/animals to bacteria that possibly acquire *ant(3'')*-Ia from plant

Except for the persistence of DNA, several other factors should be taken into consideration when assessing the probability of the transfer to occur and its possible consequences: 1) The amount of *ant(3'')*-Ia DNA is very low compared to the total ingested DNA from food and the *ant(3'')*-Ia gene does not contain any special features that would render it more resistant to digestion in the gastro-intestinal tract than any other DNA; 2) the *ant(3'')*-Ia gene is common in Gram-negative bacteria (Shaw *et al*., 1993), including normal gut bacteria (see also Chapter 2.2.).

4.2. Environmental risk assessment

4.2.1. Potential effects on plant fitness

The presence of the streptomycin resistance gene in the transgenic plant does not give the plant a selective advantage, because the gene is not expressed. Therefore effects on plant fitness, for example as a result of the presence or application of streptomycin, will not occur.

4.2.2. Potential for gene transfer

4.2.2.1. Plant to plant gene transfer

If the *ant(3'')*-Ia gene would move from a crop plant to related plants by pollination, its effects on the recipient plant would not be expected to be different from those on the donor plant. Thus the transfer of *ant(3'')*-Ia to other crops or related weeds will have no adverse environmental effects.

4.2.2.2. Plant to bacteria gene transfer

The *ant(3'')*-Ia gene is of bacterial origin, and streptomycin-resistant bacteria are present in all soils investigated (van Overbeek *et al*., 2002, Chapter 2.2.). Many of the genes conferring streptomycin resistance are transferred between bacteria because they reside on transposons and often on transmissible plasmids (van Overbeek *et al*., 2002, Chapter 2.1.2.). Some calculations are shown for *aph*(3')-IIa, in Nap *et al*. (1992) and Redenbaugh *et al*. (1994). These calculations are assumed not to be different for the transfer of the *ant(3'')*-Ia gene from GM plants to bacteria.

A number of reports in scientific literature describe the outcomes of experiments in which the ability of the *ant(3'')*-Ia gene present within experimental GM crops to be transferred to bacterial recipients has been tested. De Vries *et al*. (2004) used transplastomic tobacco plants, *i.e.* with chloroplast DNA containing the *ant(3'')*-Ia gene. It was considered that because of the high number of chloroplasts in plant cells, the transgene will occur in a high copy number. In the *in vitro* experiments, the recipient was *Acinetobacter*, a soil bacterium known to be competent for DNA uptake, containing plasmids with or without the *ant(3'')*-Ia gene and sequences corresponding to the chloroplast sequences flanking the *ant(3'')*-Ia gene. In the *in vivo* situation, bacteria such as *Acinetobacter* can act as opportunistic pathogens in plants that have already been infected by the plant pathogen *Ralstonia solanacearum*. It was thus observed that transformation with DNA from tobacco leaves only occurred in the instance of sequence homology, *i.e.* if *ant(3'')*-Ia and chloroplast sequences were already present within the recipient plasmid. These findings are in line with other reports in the literature on the requirement for the presence of homologous flanking sequences for successful transformation with *ant(3'')*-Ia-gene-containing donor DNA of *Acinetobacter* containing recipient plasmids, as reported by Kay *et al*. (2002) and Monier *et al*. (2007). Furthermore, Monier *et al*. (2007) observed that for successful transfer of DNA fragments containing the intact *ant(3'')*-Ia gene, the minimum length of the sequences in the recipient that need to be homologous to sequences flanking the *ant(3'')*-Ia gene increased concurrent with an increase in size of the DNA fragment to be transferred (Monier *et al*., 2007, Chapter 2.1.1.3.).

As mentioned above, various publications have described experiments in which model plasmids or transgenic plants containing the *ant(3'')*-Ia gene flanked by chloroplast DNA showed that the transfer was only possible if there were identical sequences of sufficient length in both the donor and recipient, flanking the DNA containing the *ant(3'')*-Ia gene in the donor. These experiments nonetheless represent a "worst case" scenario because of the high copy numbers of chloroplasts within plant cells (and thus also the number of gene copies) and the prokaryotic evolutionary origin of chloroplasts. The transfer of the *ant(3'')*-Ia gene through natural transformation from nuclear DNA may therefore be even less likely, if it occurs at all. Moreover, the *ant(3'')*-Ia gene appears to be present in a wide range of bacteria in all environments investigated (Chapter 2.2.), which apparently relates to its occurrence on integrons and/or transposons that may be prone to horizontal transfer between bacteria (Chapter 2.1.2.).

5. Prevalence of the *ant(3'')*-Ia gene

A misconception occasionally associated with the introduction of GM plants for food and feed is that the only source of resistance genes is the GM plant. However, the *ant(3'')*-Ia gene originates from common bacteria, and is not novel to the environment, the food and the feed supply (Chapter 2.1.2.). Like other streptomycin resistance genes, the *ant(3'')*-Ia gene is demonstrated to be distributed in bacteria from a range of European environmental habitats (van Overbeek *et al*., 2002) and in bacteria associated with food, feed, humans and animals (Roe *et al*., 2003; Nandi *et al*., 2004; Enne *et al*., 2008; Srinivasan *et al*., 2007).

6. Conclusion

The *ant(3'')*-Ia gene is not expressed in the GM cotton plants, and therefore there is no need to take the safety of the ANT(3'')-Ia protein into consideration. The safety of the presence of the *ant(3'')*-Ia gene in the cotton plants was considered. The exposure of humans and animals to the gene via food and feed is very low due to degradation of DNA during processing and in the gastrointestinal tract.

Appendix C

ANTIBIOTIC RESISTANCE MARKER GENES PRESENT IN THE GM PLANTS IN THE APPLICATIONS UNDER REGULATION 1829/2003 AND IN THE NOTIFICATIONS UNDER DIRECTIVE 2001/18/EC RECEIVED BY EFSA

GM plants containing intact copies of antibiotic resistance marker genes

Product	Antibiotic resistance marker genes[a]	Opinion by the GMO Panel
Recombinant human intrinsic factor extracted from GM Arabidopsis thaliana with vitamin B12	*npt*II in the Arabidopsis thaliana from which the human intrinsic protein is extracted	
Cotton LLCotton25 x MON15985	*npt*II, *aad*A from event MON 531 which was retransformed to develop event MON 15985	
Cotton MON 531	nptII, aadA	
Cotton MON15985	*npt*II, *aadA* from event MON 531 which was retransformed to develop event MON 15985	
Cotton MON1445	nptII, aadA	
Cotton MON88913 x MON15985	*npt*II, *aadA* from event MON 531 which was retransformed to develop event MON 15985	
Maize MON863	nptII	EFSA, 2004b, c
Maize MON863 x MON810	*npt*II from event MON 863	EFSA, 2005b; EFSA, 2004b, c
Maize MON863 x MON810 x NK603	*npt*II from event MON 863	EFSA, 2005d
Maize MON863 x NK603	*npt*II from event MON 863	EFSA, 2005c
Potato EH92-527-1	nptII	EFSA, 2006b, c

[a]In all cases, *npt*II served as plant selectable marker, *aadA* as bacterial selectable marker.

GM plants containing partial copies of antibiotic resistance genes have been addressed in the following EFSA opinions: EFSA, 2007b; 2006b, c, d; 2005b, c, d; 2004b, c.

Appendix D

MINORITY OPINIONS

1. Minority Opinion expressed by Dr. Christophe Nguyen-Thé.

I object to the following text: "*Notwithstanding these uncertainties, the current state of knowledge indicates that adverse effects on human health and the environment resulting from the transfer of these two antibiotic resistance genes from GM plants to bacteria, associated with use of GM plants, are unlikely*", in the Summary and Conclusions of the Opinion.

I propose that the above conclusion be deleted and replaced by the following conclusions:

- The current state of knowledge, notwithstanding the uncertainties highlighted in the present Opinion, indicates that the transfer of antibiotic resistance genes from GM plants to bacteria is unlikely.
- Should such transfer occur, any adverse effects on human health and the environment, cannot yet be assessed, but it would be imprudent to regard resistance to any antibiotic as being of little or no relevance to human health.

2. Minority Opinion expressed by Dr. Ivar Vågsholm.

2.1. Content

I object to the following text: "*Notwithstanding these uncertainties, the current state of knowledge indicates that adverse effects on human health and the environment resulting from the transfer of these two antibiotic resistance genes from GM plants to bacteria, associated with use of GM plants, are unlikely*", in the **Summary and Conclusions of the Opinion**.

It should be changed to the following conclusions:

- The transfer of antibiotic resistance markers genes from GM plants to bacteria, appear to be either not occurring, or occurring below the detection limits or at very low levels (10^{-9} probability of a transfer per exposure) as outlined in Table 1.
- However, given the magnitude and multitude of exposures from the foreseen use of GM plants with antibiotic resistance marker genes for food and feed purposes; it appears that the cumulative probability of transfer could range from unlikely to high.
- To be able to determine whether the risk is high, low or unlikely, one needs to be able to estimate probabilities of antibiotic gene transfer from GM plants to bacteria. These probabilities are below the detection limits for the studies reported.
- At the global level adverse effects on public health and environment resulting from this possible transfer cannot be assessed.

The following **Recommendation** should also be included:

• A meta-analysis of all the studies of gene transfer from GM plants to bacteria should be done using a Bayesian approach to produce better estimates of and credibility intervals for the probability of such a transfer.

2.2. Argumentation

2.2.1. Context

• The emerging pandemic of antibiotic resistance poses a serious threat to public and animal health.

• Once bacteria with antibiotic resistance genes have achieved full viability, they will behave like other contagious bacterial diseases.

• Antibiotics such as kanamycin, streptomycin that have been less frequently used for decades, often due to their severe side effects, are now being put into use again as the 2nd or last line drug against serious contagious diseases and life threatening infections, e.g. resistant *Mycobacterium tuberculosis* infections.

• It ought to be recalled that alternatives to the use of antibiotic resistance marker genes exist.

2.2.2. Considerations on the report

The current report is an excellent summary of current state of knowledge.

Key finding 2nd last paragraph of 2.1.1.4: "*Current scientific evidence indicates that the transfer from GM plants into bacteria and the stable integration of antibiotic resistance marker genes from GM plants to bacteria either does not occur or, if it has occurred, it has been below the limit of detection in all the experiments performed in the different ecosystems involved in the process. This conclusion is also supported by inference from bioinformatics studies*".

2.2.3. Illustration of the assessment by the following calculations

The probability of gene transfer is below 10^{-9} according to Table 1.

Assuming that 1 kg is the content in the human gastro-intestinal (GI) system, there are approximately 10^{13} bacteria (10^{10} bacteria per ml of GI content, and a total content of 1000 ml). Hence, the number of bacteria where transfer of resistance marker genes from plants has occurred could range from 0 to 10,000 ($10^4 = 10^{-9} * 10^{13}$).

Assuming that antibiotic resistance marker gene transfer could only could happen in the mouth with approximately 1 ml of content, then the number of bacteria where transfer of antibiotic resistance genes has occurred could range from 0 to 10 per person consuming gene modified plants.

Considering that more than a billion people will consume GM plants per year on several occasions, the number of bacteria where such transfer of resistance takes place could range from 0 to more than 10^{10} (based on such gene transfer taking place solely in the mouth).

Similar calculations can be done for food producing animals (cattle, sheep, goats, pigs and poultry).

Annex 2

European Food Safety Authority

The EFSA Journal (2009) 1035, 1-9

SCIENTIFIC OPINION

Consequences of the Opinion on the Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants on Previous EFSA Assessments of Individual GM Plants [1]

Scientific Opinion of the Panel on Genetically Modified Organisms (GMO)

(Question No EFSA-Q-2008-04977)

Adopted on 25 March 2009

PANEL MEMBERS[*]

Hans Christer Andersson, Salvatore Arpaia, Detlef Bartsch, Josep Casacuberta, Howard Davies, Patrick du Jardin, Niels Bohse Hendriksen, Lieve Herman, Sirpa Kärenlampi, Jozsef Kiss, Gijs Kleter, Ilona Kryspin-Sørensen, Harry A. Kuiper, Ingolf Nes, Nickolas Panopoulos, Joe Perry, Annette Pöting, Joachim Schiemann, Willem Seinen, Jeremy B. Sweet and Jean-Michel Wal.

SUMMARY

The European Commission has requested the European Food Safety Authority (EFSA) to deliver a scientific opinion on the use of antibiotic resistance genes as marker genes in genetically modified (GM) plants. The scientific opinion should take account of the previous opinion and the statement on the use of antibiotic resistance marker genes in GM plants and explain the rationale leading to the conclusion of whether the use of each particular antibiotic resistance marker gene is likely or not to have adverse effects on human health and the environment. The opinion should also serve as a basis for the case-by-case safety assessment of each GM plant and its processed products. These aspects have been addressed in the EFSA scientific opinion on the use of antibiotic resistance genes as marker genes in genetically modified plants available at http://www.efsa.europa.eu/EFSA/ScientificPanels/GMO/efsa_locale-1178620753812_GMOOpinions455.htm.

In addition, the European Commission requested EFSA to indicate the possible consequences of this new opinion on the previous EFSA assessments of individual GM plants containing

[1] For citation purposes: Scientific Opinion of the Panel on Genetically Modified Organisms (GMO) on a request from the European Commission on the consequences of the opinion on the use of antibiotic resistance genes as marker genes in genetically modified plants on previous EFSA assessments of individual GM plants. *The EFSA Journal* (2009) 1035, 1-9

[*] (minority opinion) This opinion is not shared by 0 members of the Panel. / (conflict of interest) 0 members of the Panel did not participate in (part of) the discussion on the subject referred to above because of possible conflicts of interest.

antibiotic resistance marker genes. This aspect is addressed in this scientific opinion of the GMO Panel.

The GMO Panel has issued previously scientific opinions about the safety of two GM plant events that contain the *aph(3')*-IIa gene (*npt*II), i.e. maize MON 863 and hybrids and starch potato EH92-527-1. In the light of the new EFSA scientific opinion "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants", the GMO Panel is of the opinion that its previous assessments on GMOs containing this antibiotic resistance marker gene are in line with the risk assessment strategy described in the above opinion, and that no new scientific evidence has become available that would prompt the Panel to change its previous opinions.

Key words: Directive 2001/18/EC, Regulation 1829/2003, GMOs, GM plants, antibiotics, antibiotic resistance marker genes, safety, food safety, human health, environment, *npt*II.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The Health & Consumer Protection Directorate-General (DG SANCO and DG ENV) of the European Commission gave a mandate to EFSA on 14 May 2008 for a 'consolidated opinion on use of antibiotic resistance marker genes used as marker genes in genetically modified plants'. The Commission letter annexed correspondence from Greenpeace (13 February 2008 and 13 September 2007) and from the Danish authorities (14 March 2008) related to the antibiotic resistance marker gene issue.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

According to Article 29 of Regulation (EC) No 178/2002[2], EFSA was requested:

1. To prepare a consolidated scientific opinion taking into account the previous opinion and the statement on the use of antibiotic resistance marker genes in GM plants intended or already authorised to be placed on the market and their possible uses as food and feed, for import and processing and for cultivation.

This Opinion should explain the rationale leading to the conclusion of whether the use of each particular antibiotic resistance marker gene is likely or not to have adverse effects on human health and the environment and outline the reasoning leading to each conclusion. The opinion should also serve as a basis for the case-by-case safety assessment of each GM plant and its processed products.

2. To indicate the possible consequences of this new opinion on the previous EFSA assessments on individual GM plants containing antibiotic resistance marker genes.

EFSA was asked to work in close collaboration with the European Medicines Agency (EMEA) and any other appropriate scientific institutes having recognised international expertise in the field of antibiotic resistance in order to characterise the use and importance of the antibiotics for which these genes encode resistance.

Given the need to proceed in a timely manner with the outstanding applications/notifications of products containing antibiotic resistance marker (ARM) genes, the Commission initially set a deadline for the opinion to not later than 30 September 2008. From the request by EFSA, the deadline was extended until March 2009.

APPROACH TAKEN TO ANSWER TO THE TERMS OF REFERENCE

After receiving this request from the Commission EFSA has asked the GMO Panel and the BIOHAZ Panel to issue jointly a scientific opinion on the use of antibiotic resistance genes as marker genes in genetically modified plants, addressing the first point of the terms of reference. That opinion is published together with this opinion and is available at http://www.efsa.europa.eu/EFSA/ScientificPanels/GMO/efsa_locale-1178620753812_GMOOpinions455.htm. The second point of the terms of reference - indicating the possible consequences of this new opinion on previous assessments of

[2] OJ L 31, 28.1.2002, p. 1.

individual GM plants containing antibiotic resistance marker genes issued by the GMO Panel - is addressed in this scientific opinion by the GMO Panel.

ASSESSMENT

1. Introduction

Following a request from the European Commission to the European Food Safety Authority (EFSA) the Panel on Genetically Modified Organisms (GMO) and the Panel on Biological Hazards (BIOHAZ) were asked to deliver a scientific opinion on the use of antibiotic resistance genes as marker genes in genetically modified (GM) plants. The scientific opinion should take account of the previous opinion and the statement on the use of antibiotic resistance marker genes in GM plants intended or already authorised to be placed on the market and their possible uses as food and feed, for import and processing and for cultivation. It was asked whether the opinion could explain the rationale leading to the conclusion of whether the use of each particular antibiotic resistance marker gene is likely or not to have adverse effects on human health and the environment and outline the reasoning leading to each conclusion. The opinion should also serve as a basis for the case-by-case safety assessment of each GM plant and its processed products. These aspects have been addressed in the scientific opinion of the Panel on Genetically Modified Organisms (GMO) and the Panel on Biological Hazards (BIOHAZ) on the use of antibiotic resistance genes as marker genes in genetically modified plants available at http://www.efsa.europa.eu/EFSA/ScientificPanels/GMO/efsa_locale-1178620753812_GMOOpinions455.htm.

In addition, the European Commission requested EFSA to indicate the possible consequences of this new opinion on the previous EFSA assessments on individual GM plants containing antibiotic resistance marker genes. That aspect is addressed in this scientific opinion of the GMO Panel.

2. Statement

The GMO Panel has issued previously scientific opinions about the safety of two GM plant events that contain the *aph(3')*-IIa gene (*npt*II), *i.e.* maize MON 863 and its hybrids and starch potato EH92-527-1 (Table 1). In the light of the new EFSA scientific opinion "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants", the GMO Panel is of the opinion that its previous assessments on GMOs containing this antibiotic resistance marker gene are in line with the risk assessment strategy described in the above-mentioned opinion, and that no new scientific evidence has become available that would prompt the Panel to change its previous opinions.

Table 1. GM plants containing intact copies of antibiotic resistance marker genes in applications submitted under Regulation (EC) No 1829/2003 and in the notifications under Directive 2001/18/EC received by EFSA and for which there is an opinion adopted by the GMO Panel. [(a)]

Product	Antibiotic resistance marker gene [(b)]	Opinion by the GMO Panel
Maize MON863	*npt*II	EFSA, 2004a, b
Maize MON863 x MON810	*npt*II from event MON 863	EFSA, 2005a; EFSA, 2004a, b
Maize MON863 x MON810 x NK603	*npt*II from event MON 863	EFSA, 2005c
Maize MON863 x NK603	*npt*II from event MON 863	EFSA, 2005b
Potato EH92-527-1	*npt*II	EFSA, 2006a, b

[(a)] GM plants containing partial copies of antibiotic resistance genes have been addressed in the following EFSA opinions: EFSA, 2007; 2006a, b, c; 2005a, b, c; 2004a, b.
[(b)] In all cases, *npt*II served as plant selectable marker.

CONCLUSIONS

In the light of the new EFSA scientific opinion "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants", the GMO Panel is of the opinion that its previous assessments on GMOs containing this antibiotic resistance marker gene are in line with the risk assessment strategy described in the above-mentioned opinion, and that no new scientific evidence has become available that would prompt the Panel to change its previous opinions.

DOCUMENTATION PROVIDED TO EFSA

1. Letter from DG SANCO and DG ENV, dated 14 May 2008, concerning the mandate for the use of antibiotic resistance marker (ARM) genes used as marker genes in genetically modified plants (ref. SANCO/E1/SP/pm (2008) D/510274).
2. Enclosure 1. May 2008. Submitted by European Commission.

   Letter from Greenpeace to the Commissioner for Health and Food Safety, dated 13 February 2008, concerning the authorisation of GM BASF potato EH92-527-1 / Agriculture Council 18 February 2008.
3. Enclosure 2. May 2008. Submitted by European Commission.

   Letter from the Danish Minister for Food, Agriculture and Fisheries and Minister of Environment to Commissioner Vassiliou and Commissioner Dimas, dated 14 March 2008.
4. Enclosure 3. May 2008. Submitted by European Commission.

   Email message from Greenpeace to Commissioner Dimas, dated 13 September 2007, concerning the Institute Pasteur study on antibiotic resistance and GM plants.

REFERENCES

EFSA, 2004a. Opinion of the Scientific Panel on Genetically Modified Organisms on a request from the Commission related to the Notification (Reference C/DE/02/9) for the placing on the market of insect protected genetically modified maize MON 863 and MON 863 x MON 810, for import and processing, under Part C of Directive 2001/18/EC from Monsanto. The EFSA Journal 49, 1-25
http://www.efsa.eu.int/science/gmo/gmo_opinions/381/opinion_gmo_06_en1.pdf

EFSA, 2004b. Opinion of the Scientific Panel on Genetically Modified Organisms on a request from the Commission related to the safety of foods and food ingredients derived from insect-protected genetically modified maize MON 863 and MON 863 x MON 810, for which a request for placing on the market was submitted under Article 4 of the Novel Food Regulation (EC) No 258/97 by Monsanto. The EFSA Journal 50, 1-25.
http://www.efsa.eu.int/science/gmo/gmo_opinions/383/opinion_gmo_07_en1.pdf

EFSA, 2005a. Opinion of the Scientific Panel on genetically modified organisms on an application (Reference EFSA GMO DE 2004 03) for the placing on the market of insect protected genetically modified maize MON 863 x MON 810, for food and feed use, under Regulation (EC) No 1829/2003 from Monsanto. EFSA Journal 252, 1-23.
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EFSA, 2005b. Opinion of the Scientific Panel on genetically modified organisms on an application (Reference EFSA GMO UK 2004 06) for the placing on the market of insect-protected glyphosate-tolerant genetically modified maize MON863 x NK603, for food and feed uses, and import and processing under Regulation (EC) No 1829/2003 from Monsanto. The EFSA Journal 255, 1-21.
http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/gmo_opinion_ej255_mon863xnk603_en1,0.pdf?ssbinary=true

EFSA, 2005c. Opinion of the Scientific Panel on genetically modified organisms on an application (Reference EFSA GMO BE 2004 07) for the placing on the market of insect-protected glyphosate-tolerant genetically modified maize MON863 x MON810 x NK603, for food and feed uses, and import and processing under Regulation (EC) No 1829/2003 from Monsanto. The EFSA Journal 256, 1-25.
http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/gmo_opinion_ej256_mon863xmon810xnk603_en1,0.pdf?ssbinary=true

EFSA, 2006a. Opinion of the Scientific Panel on genetically modified organisms [GMO] related to the notification (Reference C/SE/96/3501) for the placing on the market of genetically modified potato EH92-527-1 with altered starch composition, for cultivation and production of starch, under Part C of Directive 2001/18/EC from BASF Plant Science. The EFSA Journal 323, 1-20.
http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/gmo_op_ej323_potatoeh92-527-1_en1,0.pdf?ssbinary=true

EFSA, 2006b. Opinion of the Scientific Panel on Genetically Modified Organisms on an application (Reference EFSA-GMO-UK-2005-14) for the placing on the market of genetically modified potato EH92-527-1 with altered starch composition, for production of

starch and food/feed uses, under Regulation (EC) No 1829/2003 from BASF Plant Science. The EFSA Journal 324, 1-20. http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/gmo_op_ej324_potatoeh92-527-1_en1,0.pdf?ssbinary=true

EFSA, 2006c. Opinion of the Scientific Panel on genetically modified organisms [GMO] related to the notification (Reference C/NL/04/02) for the placing on the market of the genetically modified carnation Moonlite 123.2.38 with a modified colour, for import of cut flowers for ornamental use, under Part C of Directive 2001/18/EC from Florigene. The EFSA Journal 362, 1-19. http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/gmo_op_ej362_carnation_moonlite_en1,3.pdf?ssbinary=true

EFSA, 2007. Opinion of the Scientific Panel on genetically modified organisms (GMO) on an application (Reference EFSA-GMO-NL-2005-18) for the placing on the market of the glufosinate tolerant soybean A2704-12, for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Bayer CropScience. The EFSA Journal 524, 1-22. http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/gmo_op_ej524_A2704-12Soybean_en,0.pdf?ssbinary=true

Annex 3

European Food Safety Authority

EXECUTIVE DIRECTOR

Parma, 28 April 2009
Ref RM/PB/EW(2009) – out - **3911089**

Prof. John D. Collins, Chair BIOHAZ Panel
Prof. Harry A. Kuiper, Chair GMO Panel
Prof. Vittorio Silano, Chair of the Joint WG

Subject: **EFSA reply to the mandate for a consolidated opinion on use of antibiotic resistant marker (ARM) genes used as marker genes in genetically modified plants (EFSA Mandate No M-2008-0411)**

Dear Chairs of the GMO Panel, BIOHAZ Panel and the Joint Working Group,

In 2008, EFSA received a mandate from the European Commission (DG SANCO and DG ENV) to prepare a consolidated scientific opinion on the use of antibiotic marker genes in GM plants (Ref SANCO/E1/SP/pm(2008)D/510274).

The Terms of Reference were:
- (1) to prepare a consolidated scientific opinion on the use of antibiotic marker genes in GM plants intended or already authorized to be placed on the market;
- (2) to indicate the possible consequences of this consolidated opinion on the previous EFSA assessments on individual GMOs containing antibiotic resistance marker genes.

I would like to thank the joint Working Group and both of the Scientific Panels for the scientific work in preparing the opinion.

The joint opinion adopted by the GMO and BIOHAZ Panels includes the minority opinions of two members of the BIOHAZ Panel with respect to possible adverse effects of Antibiotic Resistance Marker Genes on human health and the environment. However, these were not discussed as minority opinions by both Panels during the adoption of the opinion.

Therefore, in order to conclude the process, I would ask both the BIOHAZ and the GMO Panels to consider the following questions:

1. Do any of the issues raised in the minority opinions require further clarification of the current joint scientific opinion?
2. If yes, can this be done without further scientific work?
3. If further work is required, what might the nature of this work be?

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In addressing these questions, the two Panels will be supported by EFSA staff and the Joint WG which could meet in the coming weeks. Since both the GMO and BIOHAZ Panels have their plenary meetings in Parma on 27-28 May 2009, a joint discussion with both Panels can also be organised.

Yours sincerely,

Catherine Geslain-Lanéelle

Cc: Mr Robert Madelin, Director General, DG Health and Consumers
Mr Karl Falkenberg, Director General, DG Environment

Annex 4

Prof. John D. Collins, Chair of EFSA's BIOHAZ Panel
Dr. Harry A. Kuiper, Chair of EFSA's GMO Panel
Prof. Vittorio Silano, Chair of the Joint GMO – BIOHAZ WG

Parma, 25 May 2009

Ms Catherine Geslain-Lanéelle
Executive Director of EFSA

Subject: **EFSA reply to the mandate for a consolidated opinion on use of antibiotic resistant marker (ARM) genes used as marker genes in genetically modified plants (EFSA Mandate No M-2008-0411)**

Dear Ms Geslain-Lanéelle

Thank you for your letter dated 29th of April, 2009 (Ref RM/PB/EW(2009) – out – 3911089) in connection with a mandate from the European Commission (DG SANCO and DG ENV) to prepare a consolidated scientific opinion on the use of antibiotic marker (ARM) genes in GM plants (Ref SANCO/E1/SP/pm(2008)D/510274), acknowledging the work done and thanking the GMO Panel, the BIOHAZ Panel, and the members of a joint GMO-BIOHAZ Working Group.

The joint GMO-BIOHAZ Working Group prepared a consensual draft scientific opinion entitled "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants", which was presented to and jointly adopted by the GMO and BIOHAZ Panels. This Scientific Opinion includes two minority opinions (*The EFSA Journal* (2009) 1034, 1-81).

To finalize the work done with regard to this opinion you asked the GMO and BIOHAZ panels to consider several questions:

1. Do any of the issues raised in the minority opinions require further clarification of the current joint scientific opinion?
2. If yes, can this be done without further scientific work?
3. If further work is required, what might the nature of this work be?

Following an in-depth consultation amongst ourselves, we would like to respond to your questions as follows: the scientific issues related to the minority opinions have already been extensively considered during the preparation of the joint Scientific Opinion and the formulation of the conclusions therein. Thus, from a scientific perspective, further

clarification of this joint Scientific Opinion is not required, nor is further scientific work needed at this time.

Considering several ongoing activities by EFSA on antimicrobial resistance, we would like to suggest that the outcomes of this opinion as well as relevant opinions from other panels be analyzed and exploited in a comprehensive context aimed at optimal risk assessment of antimicrobial resistance.

Yours sincerely,

Prof. John D. Collins
Chair of EFSA's BIOHAZ Panel

Dr. Harry A. Kuiper
Chair of EFSA's GMO Panel

Prof. Vittorio Silano
Chair of the Joint GMO – BIOHAZ WG