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NORWEGIAN SCIENTIFIC COMMITTEE FOR FOOD SAFETY

Report from an *Ad Hoc* Group appointed by the Norwegian Scientific Panel on Genetically Modified Organisms and Panel on Biological Hazards.

Document title:

An assessment on potential long-term health effects caused by antibiotic resistance marker genes in genetically modified organisms based on antibiotic usage and resistance patterns in Norway.

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Abbreviations

Amp^R: ampicillin-resistant
AR: antibiotic resistance
ARM: antibiotic resistance marker
ATP: adenosine triphosphatase
EFSA: European Food Safety Authority
Epsps: 5-enolpyruvylshikimate-3-phosphate synthase; an enzyme which confers resistance to the herbicide glyphosate
GIT: gastrointestinal tract
GM: genetically modified
GMO: genetically modified organism
GMP: genetically modified plant
HGT: horizontal (lateral) gene transfer
SGF: simulated gastric fluid
SIF: simulated intestinal fluid

Executive summary

Usage of antibiotics selects for resistant bacteria, resulting in reduced treatment options, and increased morbidity and mortality from microbial infections.
Development of resistance in susceptible bacteria can occur through spontaneous mutation or horizontal gene transfer (HGT). Our current understanding of resistance development in bacterial pathogens is more descriptive than predictive in nature. That is, whereas the acquisition or development of new resistance determinants in bacteria can be retrospectively described relatively easily at the molecular, species and geographical distribution levels, the initial horizontal transfer events, the resistance gene donor, and the environmental location and conditions that produced the first generation of the resistant bacteria remain largely unknown. Without this latter knowledge and without a clear understanding of directional selection and genetic drift in natural bacterial populations, it is impossible to predict accurately further resistance development occurring through HGT.
Some of the antibiotic resistance marker (ARM) genes used in the production of genetically modified organisms (GMO) encode resistance to antibiotics in clinical and veterinary use. Thus, concerns have been raised that the large-scale release of such genes in commercialized GMOs may increase the rate of, and broaden the locations where, bacteria horizontally acquire resistance genes.

The European Food Safety authority (EFSA) opinion (2004) identifies two main criteria that can be adopted to approximate and supplement the lack of a direct predictive capability of the effect of ARM gene usage in GMOs. These two criteria are 1) identification of the current natural reservoirs of similar resistance genes (section 3.3.3), and 2) identification of selective conditions favouring bacteria that have acquired ARM genes (section 3.3.4). These criteria enable a comparative assessment of the relative contribution of ARM genes from GMOs to the overall AR gene reservoir in the environment, and to resolve whether rare bacterial transformants that have acquired ARM genes are likely to undergo positive selection leading to clinically troublesome populations.

The EFSA opinion categorizes the most frequently used ARM genes into 3 groups. The EFSA panel considers that there is no rationale to restrict or prohibit the use of ARM genes in Group 1, (*npt*II, *hpt*) that the use of ARM genes in Group 2 (*cat*, *str*, $bla_{TEM\text{-}1}$) should be restricted to field trial purposes only, and that ARM genes in Group 3 (*nptIII*, *tetA*) should not be present in genetically modified plants (GMPs) to be placed on the market, or in GMPs used for experimental field trials. The opinion of the EFSA panel does not necessarily reflect the more precautionary motivated regulations of ARM genes for commercial use in food and feed in Norway.

The Norwegian Scientific Panel on Genetically Modified Organisms and the Panel on Biohazards appointed an *ad hoc* group to conduct a scientific risk assessment on the use of ARM genes in GMOs, as outlined under the terms of reference (section 1). The *ad hoc* group observes that there are differences between European countries in bacterial resistance levels and usage levels of antibiotics, representing the two main criteria used by the EFSA panel for classification of the ARM genes. The *ad hoc* group has therefore focused on conditions of particular relevance for the long-term effects of introduction of ARM genes to Norway, and Norwegian levels of antibiotic

resistance and antibiotic usage patterns.

Literature survey indicates that only few data are available on the prevalence of the *npt*II gene in Norway. The limited data suggest the Group 1 gene *npt*II is only present at low proportions in bacteria from natural and clinical environments in Norway. The *npt*II gene has been found in manure and sewage in a limited number of samples from the Netherlands and Germany. However, since past and present agricultural usage of antibiotics varies considerably between countries, these observations need to be documented in Norwegian environments. Given the current usage pattern of aminoglycosides in Norway, and the low level of phenotypic resistance to aminoglycosides in pathogenic bacteria in Norway, the large scale introduction of the *npt*II gene in food and feed could pose a risk to animal health. More information on *npt*II gene copy number in relevant Norwegian environments may alter this observation. Little information is available on the distribution and ecology of the *hpt* gene. However, due to the limited clinical impact of this gene, in both medical and veterinary settings, the *ad hoc* group has not identified specific concerns on the usage of this gene as an ARM.

The Group 2 gene, $bla_{TEM\text{-}1,}$ is unlikely to be present at high concentrations in natural environments in Norway. However, clinical studies of *E. coli* and other enterobacterial isolates suggest that the $bla_{TEM\text{-}1}$ gene is present in considerable quantities in the intestinal system of humans. Despite the apparently high prevalence of this gene among bacteria that are widely distributed in anthropogenic environments, the antibiotics to which the $bla_{TEM\text{-}1}$ gene confers resistance are widely used in clinical and veterinary treatment of infections in Europe, including Norway. This suggests that there is a narrow species distribution of the resistance gene and that the gene is present within these few host bacterial populations in high proportions. The *str* gene seems to have a broad distribution among various habitats in Europe, although copy number estimates are rarely provided. Little information is available on the *cat* gene distribution among species and environments in Europe. The broad usage and utility of the antibiotics to which the $bla_{TEM\text{-}1}$ gene confers resistance, combined with the observation of emerging ampicillin resistance in previously susceptible species, suggests that precautions should be taken against the dissemination of AR genes in environments that are selective for bacterial transformants carrying specific ARM genes.

It is unclear how the EFSA opinion and assessment (EFSA, 2004) define and distinguish quantitatively the prevalence of the genes in relation to the group categorization and assessment made. It is also unclear if the prevalence argument is based on considerations of the ARM copy number only, or if the relative presence of ARM gene homologues among relevant clinical isolates in different countries is also considered. The lack of relevant data and quantitative definitions easily leads to subjective and contested interpretations of the relevant usage level and the resistance level for the group categorization. The *ad hoc* group recommends strengthening of public research efforts to resolve some of the major knowledge gaps identified for the direct and indirect effects of ARM genes (and other food-derived DNA) on human health (section 5). This is necessary to develop scientific consensus on the quantitative definitions of the categorization criteria as presented in the EFSA opinion (EFSA, 2004). Furthermore, data on the specific usage of the relevant antibiotics and corresponding resistance patterns in Europe needs to be systematically collected to improve ARM gene risk assessment further and to enable epidemiological monitoring of ARM gene homologues.

1. Terms of Reference

In a letter dated 11 February 2005, the Norwegian Food Safety Authority requested the Norwegian Scientific Committee for Food Safety to perform an assessment of the risk to human health and the environment on the use of antibiotic (antimicrobial) resistance (AR) genes as marker genes for genetically modified organisms (GMOs). The assessment should identify possible knowledge gaps and uncertainties related to such risk, and also consider the need for antibiotic resistance marker (ARM) genes in GMOs, possible alternatives to ARM genes, and possible risks connected to the alternatives. Finally, the assessment should consider the resistance situation in Norway, as compared to the situation in other European countries, and the use of antibiotics in human and veterinary medicine in Norway.

2. Introduction

The introduction of genetic material into the cells and genomes of higher organisms is only infrequently successful. It is therefore necessary to have the means to discriminate between cells that have acquired the intended genetic insertion, versus the large majority of cells that remain unmodified. Selectable marker genes are used extensively in genetic engineering to allow rapid identification and selective amplification of cells that have successfully received the new genetic material. Marker genes encoding antibiotic resistance (ARM genes) are often used for higher organisms because these allow dominant selection to be included in the growth medium of the modified cells/tissues. The marker genes have no function in the product, but since their subsequent removal is difficult, they often remain in the commercialized GMO. Due to the lack of peer-reviewed data demonstrating either the long-term safety or an associated risk of ARM genes, it has been difficult to reach scientific, regulatory and public consensus on the continued use of such marker genes in commercialized transgenic plants (Nap et al., 1992, WHO, 1993, 2000, FDA, 1998, Kok et al., 1994, Kärenlampi, 1996, Salyers, 1996, Metz and Nap, 1997, Kruse and Jansson, 1997, Nielsen et al., 1998, Malik and Saroha, 1999, GM Science Review Panel, 2003).

The presence of ARM genes in commercialized GMOs does not result in an agricultural or consumer benefit. Moreover, the continual increase in resistance to antibiotics observed for most major pathogens has resulted in the Norwegian government taking a restrictive and precautionary approach on the use of ARM genes in the production of GMOs intended for release and sale in Norway. The Norwegian National Assembly has adopted two regulations that prohibit the commercial production, and import and sale of GM food (from 1 June 2002) and feed (from 7 November 2002) where inserted ARM genes are present and intact in the final product.

In Europe, the presence of ARM genes in GMOs has been controversial, partly due to the lack of specific guidelines regulating their use. Case-by-case assessments have put variable emphasis on the presence of ARM genes in GMOs and their potential health and environmental effects, if unintentionally transferred to pathogenic bacteria. Part B of Directive 2001/18/EF states that ARM genes should be taken into consideration when conducting risk assessments of GMOs

containing such genes, in particular genes expressing resistance to antibiotics in use for medical or veterinary purposes. According to the directive, ARM genes that may have adverse effects on human health and the environment should be identified and phased out. ARM genes that may confer adverse effects should be phased out before 31 December 2004, when present in GMOs intended for commercial use, and before 31 December 2008, when present in GMOs used for field trials. The directive 2001/18/EF also states that the future development of GM-plants to be placed on the market, and to be used in the production of food or feed, should aim to avoid genes that confer resistance to therapeutically relevant groups of antibiotics.

In 2004 the Scientific Panel on Genetically Modified Organisms (GMO Panel) of the European Food Safety Authority (EFSA) published an opinion on environmental and health aspects of ARM genes, particularly those already in use in genetically modified plants (EFSA, 2004). The opinion considered seven different ARM genes and classified the genes according to their assessed potential for creating increased resistance to antibiotics in human and animal pathogens after horizontal transfer. Two main criteria were used to assess the potential impact of putative transfer and positive selection of ARM genes in pathogenic microorganisms: 1) the prevalence of the ARM gene homologues in natural microbial communities, and 2) the clinical and veterinary usage levels of the antibiotics to which the specific ARM gene confers resistance. Thus, data on the amount of the relevant antibiotics used in Europe, and the resistance levels to the relevant antibiotics in the European Union, provided a main baseline for the assessment. The ARM genes were classified by the EFSA GMO panel into 3 groups:

Group 1: Genes *npt*II and *hpt,* which confer resistance to the antibiotics kanamycin/neomycin/paromycin/butirosin/gentamicin B/geneticin or hygromycin, respectively.

Group 2: Genes Cm^r *(cat)*, Amp^r (bla_{TEM-1}) and *str* (*aadA),* which confer resistance to the antibiotics chloramphenicol, or ampicillin or streptomycin/spectinomycin, respectively.

Group 3: Genes *npt*III and *tetA,* which confer resistance to the antibiotics amikacin or tetracyclines, respectively.

The EFSA GMO Panel considered that there is no rationale for restricting or prohibiting the use of ARM genes placed in Group 1. The panel recommended that the use of ARM genes in Group 2 should be restricted to field trial purposes only, and that ARM genes in Group 3 should not be present in GMPs to be placed on the market or in GMPs used for experimental field trials.

The opinion of the EFSA GMO panel is not necessarily consistent with the precautionary motivated regulations for ARM genes for commercial use in food and feed in Norway. The Norwegian Scientific Panel on Genetically Modified Organisms and the Panel on Biohazards appointed an *ad hoc* group to conduct a scientific risk assessment on the use of ARM genes in GMOs, as outlined under the terms of reference. The Norwegian *ad hoc* group decided to consider the potential health and environmental effects of ARM genes placed by the EFSA GMO panel in Groups 1 and 2 only, since the restricted use of the ARM genes in Group 3 is in line with current Norwegian legislation.

The *ad hoc* group observes that there are clear differences between European countries in

bacterial resistance levels and usage levels of antibiotics, representing the two main criteria used by the EFSA GMO panel for classification of the ARM genes. The *ad hoc* group has therefore focused on conditions of particular relevance to the long-term effects of ARM genes introduced into Norway, and Norwegian levels of antibiotic resistance and antibiotic usage patterns. The *ad hoc* group has considered only the potential effects of ARM genes present in GMPs.
Given the limited documentation of alternative marker technology, the *ad hoc* group considered the task of reviewing biosafety aspects of alternative markers to require a substantial investigation and a separate assessment and report. Hence, an assessment of alternative marker technology has not been included here.

3. Overview of environmental and health effects considered in the risk assessment

ARM genes are originally isolated from naturally occurring bacteria from species-diverse microbial communities. Although the exact locations and prevalences of these genes are often not known, microorganisms in various environments are naturally exposed to such resistance genes at variable levels. Moreover, bacteria are known to transfer antibiotic resistance genes frequently between cells and species. The large-scale release of GMOs containing ARM genes may not necessarily, therefore, introduce new antibiotic resistance genes into a particular environment. However, the GMO introduction may change the environmental persistence and concentrations, of AR gene exposure to various microorganisms, and may alter the frequency and locations of such genes. The insertion of modified ARM genes into eukaryotic chromosomes in GMOs will necessarily alter the genomic locations of ARM genes substantially from those genomic locations AR genes usually occupy in prokaryotic microorganisms. The changed genomic insertion sites and positions may alter the stability and transferability of the ARM genes. However, AR genes are known to occupy a range of genomic locations in various microorganisms, making them some of the most dynamic genetic entities known.

The *ad hoc* group has focused on the following potential effects caused by the presence of ARM genes in GMOs released in field trials or for commercial purposes:

- 3.1 Toxic, allergenic or environmental effects caused by *proteins* encoded by ARM genes.
- 3.2 Health effects caused by the uptake of intact ARM *genes* into mammalian cells.
- 3.3 Indirect effects arising from the reduced ability to treat microbial infections after horizontal transfer and amplification of ARM genes in bacteria.

In assessing the above potential effects, we have drawn upon the scientific findings and recommendations published in the EFSA opinion (EFSA, 2004). We have also independently reviewed the scientific literature to determine whether ARM genes pose risks that require further precautionary action or risk management, and whether national or regional antibiotic usage and antibiotic resistance patterns are consistent with the EFSA opinion and rationale for the proposed classification of ARM genes. It should be noted that commercial GMO developers utilizing ARM genes have certainly accumulated additional relevant knowledge on ARM genes that is not available in the scientific literature. Since such information is often confidential and not made

available to open peer-review, we have been unable to include these studies in our assessment. Finally, we have identified knowledge gaps and areas for further research in order to strengthen the scientific basis for the risk assessment of ARM genes.

3.1 Potential toxic, allergenic or environmental effects caused by *proteins* encoded by ARM genes

Bacteria harbouring AR genes can be found in many unprocessed or processed food sources as well as in the environment. Human or animal exposure to gene products (proteins) of AR genes is thus not exclusively linked to the consumption of GMOs. The levels of natural proteins encoded by AR genes present in bacteria ingested with food remain unknown. The large-scale usage of GMOs, some with constitutively expressed ARM genes, may result in higher levels of ARM gene products being present in GM-food than those produced by microbes naturally-present in food. Moreover, the new cytoplasmic locations of the ARM gene products may introduce post-translational modifications of the proteins not present in microbially-produced counterparts. GMOs harbouring ARM genes will contain the gene product at variable concentrations depending on promoter type and activity, and cellular and environmental variables. High levels of ARM gene proteins can be produced in plants; e.g. up to 1% of the total cellular protein was NPTII in transplastomic plants harbouring an *npt*II gene expressed in plastids (Carrer et al., 1993). Although experimentally determined concentrations of ARM gene products are rarely available in the scientific literature, lower concentrations would be expected to be produced by ARM genes localized to the plant nucleus.

3.1.1 Acute toxicity

Group 1 gene products

NPTII. Several reviews on the safety of the *npt*II gene and corresponding protein have been published (e.g. Flavell et al., 1992, Nap et al., 1992, WHO, 1993, Redenbaugh et al., 1993, 1994). Peer-reviewed experimental data on the possible acute toxicity of the protein NPTII has been published by Monsanto (Fuchs et a., 1993b). In an acute mouse gavage study, (Fuchs et al., 1993a) a microbially-expressed NPTII protein was used. Between 100 to 5000 mg NPTII protein/kg bodyweight was gavage-fed to mice. No deleterious effects were reported for the 8 to 9 day period after NPTII administration and the study concluded that the protein poses no safety concerns. The recombinant bacterial NPTII protein has been found to be chemically and functionally equivalent to the plant (cotton, tomato and potato) expressed version (Fuchs et al., 1993a). Given that this observation is representative across recombinant *npt*II host plants, information on the effects of exposure to NPTII proteins can be derived from comparisons with naturally-occurring exposure routes and sources of NPTII proteins.

The *npt*II gene was originally found on a transposon (TN5) located on a plasmid (pJR67) from an enterobacterium (Berg et al., 1975) and has subsequently been observed in a range of enterobacterial species (Blasquez et al., 1996). Given the assumption that the *npt*II gene is expressed continually in these bacteria, and that the bacteria have a broad geographical distribution, the digestive tract of humans and animals will be naturally exposed to these

enzymes. Although the concentrations of naturally-occurring NPTII-producing bacteria and NPTII proteins in the gastrointestinal tract (GIT) of humans or animals has not been determined, health effects arising from the natural exposure to this enzyme have not been identified or reported.

Given the rapid breakdown of the NPTII protein observed in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) (Fuchs et al. 1993b), together with the hypothesized natural exposure to NPTII proteins and the absence of acute toxic effects in mice, as reported by Fuchs et al., (1993b), the *ad hoc* group concludes that the NPTII protein expressed by ARM genes is highly unlikely to pose toxic effects. Our conclusion assumes that neither potential post-translational modifications of the NPTII protein that may occur in other species and cytoplasmic conditions than those examined by Fuchs et al. (1993a), nor the protein degradation products will produce toxicological effects.

HPT. We are not aware of any peer-reviewed studies that have examined the acute toxicity of the enzyme HPT. The *ad hoc* group is not aware of any scientific studies or indications that would suggest that HPT poses acute toxicity risks in mammalian systems. Earlier assessments have not found such indications (Kärenlampi, 1996), but point to the lack of relevant scientific studies regarding: 1) the novelty of the gene product for humans; 2) detailed sequence comparisons to known toxic or allergenic proteins; and 3) detailed investigations of pleiotrophic effects i.e. the phosphorylation state of the plant cell (Kärenlampi, 1996).

Acknowledging the uncertainty and lack of relevant scientific studies, the *ad hoc* group is of the opinion that *hpt* gene products are unlikely to produce acute toxic effects.

Group 2 gene products

Group 2 enzymes are not intended for commercial use or consumption, and are not considered further here.

3.1.2. Allergy caused by exposure to ARM gene products

Group 1 gene products

NPTII. The NPTII protein has been reported to be rapidly degraded in SGF and SIF (Fuchs et al. 1993b). Degradation was observed within seconds in the SGF, and minutes in the SIF, as measured by Western blots and the enzymatic activity of the proteins was destroyed after 2-15 min incubation in SGF or SIF (Fuchs et al., 1993b). Based on the rapid digestion of the protein, the expected history of natural exposure of humans and animals to the NPTII protein without the identification of allergenic effects, and the lack of identified homology between the NPTII protein and known allergens, the authors suggested that the NPTII protein should not cause allergenicity concerns (Fuchs et al., 1993b). The reliability of *in vitro* digestion as an indicator of allergenic properties has later been questioned (Spök et al., 2004). The *ad hoc* group is not aware of any data suggesting that the Group 1 enzyme NPTII is likely to cause allergenic reactions beyond those potentially caused by naturally occurring NPTII enzymes in bacteria present in the GIT of humans and animals.

HPT. The *ad hoc* group is not aware of any peer-reviewed studies that have examined the allergenic potential of the HPT enzyme or other information that would suggest that exposure to the HPT enzyme *via* food consumption will result in allergenic reactions. Acknowledging the uncertainty and lack of relevant scientific studies, the *ad hoc* group is of the opinion that *hpt* gene products are unlikely to produce allergenic reactions.

Group 2 gene products

Group 2 enzymes are not intended for commercial use or consumption, and are not considered further here.

3.1.3. Accumulation of ARM gene products in the environment

Group 1 gene products

NPTII. It has been estimated that the concentration of NPTII protein in plants is between 3-440 ng per mg of soluble protein (see Kärenlampi, 1996). As described in section 3.1.2, the NPTII protein has been reported to be rapidly degraded in SGF and SIF (Fuchs et al. 1993b), indicating that the NPTII protein is not unusually stable to hydrolytic activities. Natural environments contain high number of saprophytic bacteria capable of degrading organic matter, including proteins (Burns and Dick, 2002).

The *ad hoc* group is of the opinion that the NPTII protein is likely to be rapidly degraded upon exposure to saprophytic bacteria in the environment, and that no biosafety relevant concerns have been identified, or suggested, in the scientific literature, regarding possible long-term accumulation of NPTII proteins in the environment.

HPT. The *ad hoc* group is not aware of any scientific studies that have examined the stability of the *hpt*-encoded proteins. The *ad hoc* group is not aware of any information that suggest the HPT proteins are likely to have characteristics that make it more stable than other proteins naturally present in bacterial cytoplasms.

Acknowledging the uncertainty and lack of relevant scientific studies, the *ad hoc* group is of the opinion that the HPT protein is likely to be rapidly degraded upon exposure to saprophytic bacteria in the environment, and that no biosafety relevant concerns have been identified, or suggested, in the scientific literature, regarding possible long-term accumulation of HPT proteins in the environment.

Group 2 gene products.

Due to the limited size and duration of field trials, the *ad hoc* group considers the potential environmental accumulation of Group 2 enzymes to be insignificant.

3.1.4 Inactivation of orally administered antibiotics by ARM gene encoded proteins present in food

Ingestion of food containing ARM genes encoding active enzymes could hypothetically lead to the inactivation of orally-administered antibiotics in the GIT. Although several theoretical assessments are available (Nap et al., 1992, Redenbaugh et al., 1993, 1994), we are not aware of any *in vivo* studies that have systematically examined the effects on antibiotic treatment in humans or animals following ingestion of food containing Group 1 ARM genes. Experimental studies are not available that detail the relative contribution of ARM gene encoded Group 1 enzymes, as compared to the enzymes produced by the indigenous microflora of humans or animals, to the inactivation and absorption kinetics of orally-administered antibiotics.

Group 1 gene products

NPTII and HPT enzyme activity in the GIT of humans. Antibiotics that may be inactivated by Group 1 enzymes (NPTII, and HPT) are not orally administered to humans in Norway, and cross-resistance to orally-administered antibiotics in Norway has not been reported.

The *ad hoc* group is of the opinion that Group 1 ARM genes will not interfere with the stability or effect of orally-administered antibiotics to humans in Norway.

NPTII and HPT enzyme activity in the GIT of domestic animals. Antibiotics inactivated by the Group 1 enzyme NPTII may be administered to animals in Norwegian husbandry. The main usage of neomycin is for the treatment of gastrointestinal infections of piglets and calves (H. Kruse, pers. comm.). The estimated usage of neomycin in Norway is 35 kg per year (NORM/NORM-VET, 2003). It is not used in Norwegian aquaculture.
A reduced effect of orally administered neomycin can be theoretically predicted to occur in husbandry given i) continued exposure to ARM gene encoded proteins present in feed and ii) survival and activity of the ARM gene encoded enzymes in the relevant parts of the GIT. The NPTII protein has been reported to be rapidly degraded in GIT simulations (Fuchs et al., 1993a). Although it is unclear if the protein has a comparably short degradation time *in vivo* when present in unprocessed food sources, no features have been identified in the protein suggesting that it will not undergo rapid degradation in the GIT of mammals. The NPTII enzyme requires Mg^{2+}, ATP and a pH range of 7-7.5 for optimal activity (Ganelin et al., 1980). It is therefore unlikely that NPTII enzymes will functional optimally in the GIT of humans or animals, even if some enzymes survive immediate digestion. Processing of food is likely to inactivate (denature, hydrolyze) substantial proportions of the NPTII proteins.

The digestive tract of higher animals is known to harbour a range of bacteria encoding enzymes inactivating antibiotics such as neomycin (e.g. Blasquez et al., 1996). Thus, the relative contribution of ARM gene products to inactivation of antibiotics intended for use in the digestive system is unclear and probably low. Experimental data from relevant animal models to resolve the hypothetical outcome of a reduced activity of orally-administered neomycin due to ARM gene usage are not available in the peer-reviewed literature. A brief description of a study on neomycin stability in feed is given in Redenbaugh et al. (1994), but lacks experimental detail and peer-review, thus excluding the incomplete information from further consideration here. Redenbaugh et al. (1993) provide theoretical calculations on the exposure of humans to the *npt*II

gene and gene products, and conclude that they are not of significant concern.

The *ad hoc* group is of the opinion that the Group 1 ARM gene encoded protein NPTII is unlikely to contribute significantly to the enzymatic inactivation of neomycin orally-administered in veterinary medicine in Norway. Thus, we consider the risk related to the presence of ARM gene encoded NPTII proteins in animal feed to be low. However, the continued efficiency of neomycin in Norwegian veterinary medicine after possible ARM gene introductions, must also be considered in relation to potential selection of horizontal transfers of ARM genes into pathogenic bacteria, as discussed in section 3.3.

Group 2 gene products

Group 2 ARM genes will not be used for commercial purposes and only a few field releases are expected for these ARM genes in Norway, until their prohibition on 31 December 2008. Since Group 2 ARM genes will not be used in commercial products, the *ad hoc* group has not considered these further here.

3.1.5. Conclusions from section 3.1

The limited number of experimental studies available to resolve the questions raised in sections 3.1.1 to 3.1.4 has resulted in an assessment that is based mainly on comparative experience and inference, rather than on direct experimental or epidemiological verification of the absence of effects. Most of the assumptions of a low (insignificant) risk produced by the release and exposure to ARM gene encoded enzymes, are based on inference from lack of observable effects from human and environmental exposure to naturally-occurring counterparts of the ARM gene encoded proteins. Although the natural concentrations of these resistance enzymes are often unknown, deleterious consequences from natural exposure to these enzymes have not, to the extent investigated, been reported. After reviewing the EFSA opinion paper (EFSA, 2004) and the scientific literature as described above, the *ad hoc* group considers the risk to be low for Group 1 ARM gene encoded proteins released in Norway. Due to the highly limited release of Group 2 ARM gene encoded proteins expected in Norway, the *ad hoc* group does not consider that the limited release of these proteins constitutes a health or environmental risk.

3.2 Potential health effects caused by the uptake of intact ARM *genes* into mammalian cells

All food has organic origins and therefore contains variable amounts of DNA. It has been estimated that humans ingest between 0.1 to 1 g of DNA in their food per day, and cows 60 g of DNA per day (Doerfler, 2000, GM Science Review Panel, 2003). Exposure to feed-ingested DNA is thus a common feature of higher organisms. GM-DNA is not considered to differ significantly from the chemical composition and structure of other DNA molecules present in food. When compared to the overall amount of DNA ingested and the proportion of the genome of the organism that has been modified, the quantity of GM-DNA ingested is only a minor fraction. The modified genes used in GMOs are collected from a variety of organisms. Although the modified genes may be considered novel, they have been isolated, cloned and modified from naturally-occurring living organisms. Thus, the novelty of the ARM genes in commercial use is

caused by their altered genetic context and regulation, rather than by their capability of producing unique proteins with novel amino acid compositions. Conceivably, the new genetic context and altered regulation of ARM genes in GMOs may introduce properties of importance for novel expression patterns, genetic stability, and degradation products of the transgene during the normal processing of feed-derived DNA in mammals. Many recent studies have established that small proportions of feed-derived DNA can be found in the bloodstream of mammals (see below). Although a relatively recent discovery, there are no indications that transgenes should behave any differently in the intestines of mammals, or cause other effects in the bloodstream, than any other DNA present in food.

3.2.1 Uptake of ARM genes into epithelial cells

In addition to DNA present in the diet, large amounts of DNA are produced and released (upon cell death) by microorganisms present in the GIT. The GIT contents and faeces also consist of significant proportions of epithelial cells, and hence, DNA from the host organism. ARM genes present in ingested foods could hypothetically be absorbed by luminal cells in the GIT causing unknown, but potentially negative, effects. Many studies have shown that food-ingested DNA may reach, or pass, luminal cells, although the proportion of the initially ingested DNA, and the size and quality of such DNA is likely to vary considerably depending on food source and digestive system. Table 1 outlines studies examining the stability of DNA in various gut systems. It is clear that the great majority of DNA molecules are substantially degraded upon digestion. However, purified DNA, or DNA present in GM soya and GM-maize, was not fully degraded in human intestinal simulations (Martín-Orúe et al, 2002). A recent study of human volunteers, including ileostomists (i.e. individuals in which the terminal ileum has been resected and the digesta are diverted via a stoma to a colostomy bag) fed GM-soy products, reported that fragments of DNA survive the passage through the small bowel, but cannot be detected in the faeces of volunteers with an intact digestive tract (Netherwood et al, 2004). Chowdhury et al. (2003 a, b) reported that DNA ingested by pigs fed GM-maize was not totally degraded and that DNA fragments could be detected in caecal and rectal contents. Whilst all studies performed to date suggest that most DNA entering the digestive system of mammals will be degraded, there are very few quantitative studies on the size distribution and the proportion of DNA remaining in various parts of the digestive tract, or surviving digestion. Several studies suggest that mammalian cells in culture can take up free-DNA (Anker et al., 2004, and references within), and many *in vivo* studies, as described in section 3.2.2, demonstrate that feed-derived DNA can pass luminal cells to reach various tissues in mammals. The mechanisms behind such transfer need to be further resolved and we are not aware of studies that have specifically investigated the uptake of ARM genes into luminal cells of humans or animals *in vivo*.

The *ad hoc* group considers that the observations communicated in the available peer-reviewed studies (as listed in Table 1) make it probable that minor proportions of intact ARM genes present in food, will be exposed to luminal cells. The *ad hoc* group is not aware of any studies indicating that ARM genes will behave differently, or cause any other effects than those caused by the high diversity of food-derived genes naturally exposed to luminal cells. Nor is the *ad hoc* group aware of any studies indicating that the natural uptake of free, food-derived DNA molecules from the intestinal system produces a biological effect in the host. Despite the large

uncertainty regarding the biological mechanisms following DNA exposure to luminal cells, the *ad hoc* group is of the opinion that ARM genes do not differ so significantly in composition or concentration from the wide range of naturally occurring DNA present in various food sources and the indigenous microflora in the gut of humans and animals that they are likely to cause further biological effects. The *ad hoc* group recommends that further studies should be performed to clarify the basic biological mechanisms behind translocation and transport of food-derived DNA to luminal and other mammalian cells.

Table 1. Examples of peer-reviewed studies using PCR methodology to examine the fate of recombinant DNA in food during digestion.

DNA source	Model system	Reference
GM corn	Chickens	Aeschbacher et al., 2005
GM oilseed rape	Cows	Alexander et al., 2004
GM corn	Chickens	Chambers et al, 2002
GM corn	Pigs	Chowdhury et al, 2003a,b
GM corn	Calves	Chowdhury et al, 2004
GM corn	Sheep saliva, rumen fluid	Duggan et al, 2000, 2003
GM corn	Cows, chickens	Einspanier et al, 2001, 2004
GM soybean	Chickens	Jennings et al., 2003a
GM corn	Pigs	Jennings et al., 2003b
GM corn, GM soya	Human digestion simulations	Martín-Orúe et al, 2002
GM corn	Various	Nemeth et al., 2004
GM soya	Humans (ileostomists)	Netherwood et al, 2004
GM corn, GM soya	Cows	Phipps et al, 2003
GM soya	Cows' blood, milk, urine, faeces	Poms et al, 2003
GM corn	Pigs	Reuter and Aulrich, 2003
GM corn	Chickens	Tony et al., 2003
GM corn	Rats	Hammond et al., 2004, 2005
GM potato, GM corn	Rats	Wilcks et al., 2004

3.2.2 Unintended uptake and transport of ARM genes to other tissues

The possible uptake of ARM genes from the GIT of mammals may hypothetically produce unintended consequences, after dissemination of ARM genes into various tissues. The identification of the uptake and dissemination of feed-derived DNA into mammalian tissues has been reported in a series of studies by Doerfler and colleagues (Schubbert et al., 1994, 1997, 1998, Doerfler et al., 2001). After feeding foreign DNA to mice, DNA fragments were demonstrated to have been absorbed in the gastrointestinal system, and could be detected in leucocytes, spleen, liver and kidney (Schubbert et al**.** 1997). The foreign DNA was detected in spleen and liver at 18 hours after feeding. In one instance the foreign DNA could be shown to have become covalently linked to mouse DNA. The same group later found that plasmids fed to pregnant mice could be transferred to foetuses, and later detected in the brain, eye, liver and heart of the offspring (Schubbert et al. 1998). Similar observations of extensive tissue distribution of feed-derived DNA have been made in a number of subsequent studies (see also some of the studies cited in Table 1). In chicken and cattle, fragments of plant DNA have been observed in muscle, liver, spleen and kidney after feeding with corn (Einspanier et al, 2001). Hohlweg and Doerfler (2001) detected the plant-specific ribulose-1,5-biphosphate carboxylase/oxygenase gene in the liver and spleen of mice following feeding with soybean leaves. Up to 0.1% of DNA orally-administered to mice can be retrieved in the animals' blood (Doerfler, 1996). Similarly, 0.1% of orally administered DNA has been retrieved from fish blood (Nielsen et al., 2005a, b;). However, feeding experiments demonstrated that the DNA is transferred from the GIT to the blood over several hours and undergoes continuous elimination and degradation. Thus, based on available data, it has been estimated that approximately 1% of dietary DNA is absorbed from the GIT (Nielsen et al., 2005b).

Few studies have been performed on the possible distribution of ingested DNA in human tissues. Small amounts of ingested DNA have been shown to circulate in human plasma/serum (Anker and Stroun, 2000). DNA entering the bloodstream is likely degraded by DNase activity in human serum and plasma (Connolly et al. 1962, Rozenberg-Arska et al. 1984).

The *ad hoc* group is of the opinion that although major knowledge gaps exist in the general understanding of the proportion and pathways of feed-derived DNA entering the bloodstream of mammals, there is no evidence suggesting that the ARM genes in current use will create biological effects further than that of any other DNA fragment released into the GIT from food or the intestinal microflora and entering the bloodstream.

3.2.3. Conclusions from section 3.2

The lack of a detailed understanding of the uptake mechanisms, transport pathways and degradation dynamics of food-derived DNA in the bloodstream of mammals represents major knowledge gaps that warrant further research. Moreover, the lack of quantitative data on the DNA fragment size distribution in the digestive system of mammals digesting food from a variety of sources makes precise predictions of DNA exposure rates, and the relevant physical locations of DNA currently impossible. The possible interactions of ARM gene encoded proteins and their enzymatic activity with other proteins (proteome) in mammalian cytoplasms remains

unexplored and should be further clarified.

While acknowledging the various identified knowledge gaps, the *ad hoc* group is of the opinion that mammalian exposure to ARM genes should be seen in relation to the daily exposure to DNA that mammals normally experience from any ingested food-source. The *ad hoc* group is not aware of any experimental evidence suggesting that ARM genes, or any other feed-derived DNA molecules, will produce negative effects if taken up by, or expressed in, mammalian cell cytoplasms.

3.3. Indirect effects caused by a potentially reduced ability to treat microbial infections after *horizontal transfer* of ARM genes to bacteria

Horizontal gene transfer (HGT) is known to be an important contributor to bacterial evolution and adaptation, for instance through the dissemination of AR genes in bacteria of clinical importance (Davison, 1999). HGT, when combined with positive selection (see section 3.3.4), can rapidly change the genetic composition of bacterial populations. Several mechanisms for HGT are known in bacteria (conjugation, transduction, and natural transformation) and AR genes are well known to transfer both among bacteria within the same species, and between different bacterial species. AR genes are often located on mobile genetic units with higher transfer frequencies among bacterial species than for chromosomal genes. HGT frequencies are generally considered to be lower among more divergent or unrelated species and few, if any, examples exist where bacteria have acquired plant genes and retained them in their genomes over evolutionary time. Nevertheless, the plant-pathogenic bacteria, *Agrobacterium* spp. are known to transfer specific bacterial genes naturally in the opposite direction, to plant cells.

Although experimental evidence suggests bacterial genomes are naturally exposed to plant genes (Kay et al., 2002), a number of biological barriers can explain why few plant genes are retained in bacterial genomes (Thomas and Nielsen, 2005). However, several of these transfer barriers are absent in transgenes inserted in GMPs. For instance, the absence of introns, the presence of flanking vector DNA regions with high similarity to bacterial chromosomes, and the use of broad host range promoters may increase the likelihood of functional gene transfer of transgenes from GMPs to bacteria, as compared to transfer of other wild-type plant genes (Nielsen et al., 1998, Nielsen, 2003). The large-scale release of ARM genes in GMOs can hypothetically result in an undesired increase in the exposure level of bacteria to AR genes, as well as broadening of the locations and routes of exposure. Furthermore, the presence of flanking cloning vector sequences and the replacement of native promoters may alter the recombination potential of ARM genes in bacteria. Thus, when combined with positive selection of ARM gene harbouring bacteria, this "worst case" scenario could result in an increase in the occurrence of resistant bacteria.

Development and spread of AR is an increasing global problem. Our understanding of the phenomenon is more descriptive than predictive. Whereas the bacterial acquisition of new resistance determinants can be retrospectively described relatively easily at the molecular, species and geographical distribution levels, the initial horizontal transfer events, the resistance gene donor, and the environmental location and conditions that produced the first generation of the resistant bacteria remains largely unknown. Without this latter knowledge and a thorough

understanding of the complex patterns of directional selection and genetic drift in natural bacterial populations, it is difficult to predict accurately further resistance development occurring through HGT and directional selection.

The limited positive selection of bacteria carrying AR genes *via* the restricted use of antibiotics and physical isolation are currently considered the best methods to limit the spread of bacteria with acquired resistance. Thus, understanding the population dynamics of existing or newly generated resistant bacteria is essential to determine their long-term effects (Nielsen and Townsend, 2001, 2004).

The *ad hoc* group has considered the following aspects of the potential acquisition of ARM genes in bacteria:

- 3.3.1 Mechanisms that could mediate horizontal transfer of ARM genes and their activity in relevant environments.
- 3.3.2 Experimental studies seeking to clarify the occurrence of horizontal transfer of ARM genes from GMOs.
- 3.3.3. Resistance mechanisms and prevalence of ARM gene homologues in bacterial communities.
- 3.3.4. Selective conditions favouring bacteria harbouring ARM genes.

3.3.1 Mechanisms that could mediate horizontal transfer of ARM genes and their activity in relevant environments

The successful uptake and long-term persistence of ARM genes or any other foreign DNA within bacterial cells requires a number of steps:

i) release of intact ARM genes from the cytoplasm of the GMO,
ii) persistence of intact ARM genes in the environment,
iii) exposure of the relevant species of competent bacteria to the ARM genes,
iv) indiscriminate uptake of ARM genes by the bacteria,
v) integration of the translocated ARM genes into a bacterial replicon to ensure stability over generations,
vi) expression of the ARM genes in order to produce a selectable bacterial phenotype with new resistance characteristics, and
vii) directional selection of rare bacterial transformants to produce population sizes that are clinically observable.

Below, some aspects of the DNA release, persistence and uptake steps are discussed with particular focus on environments of relevance to GMOs and ARM genes.

Release of intact ARM genes from GMOs. Several studies have monitored the persistence and stability of ARM genes in GMPs under various environmental conditions (Table 2). All the published studies indicate a gradual decrease in the quantity and quality of DNA over time. Widmer et al. (1997) reported that the *npt*II gene could be detected for up to 137 days in composted potato litter. Paget et al. (1998) used PCR to detect the *aacC*1 gene in tobacco litter

for up to 1 year. Gebhard and Smalla (1999) found positive PCR signals of DNA containing *npt*II genes from soil samples for up to 2 years after the initial farming of transgenic sugar beet (*Beta vulgaris)*. Hay et al. (2002) could detect PCR amplifiable DNA fragments for up to 4 months from transgenic poplar leaves incubated in soil. Thus, although few quantitative estimates are available, DNA molecules from plants have been detected in soil for extended periods after harvest (Table 2). Although the biochemical conditions and physical locations enabling plant DNA to persist in agricultural environments is often unclear, the published observations suggest that minor proportions of the ARM genes present in growing or decomposing GMPs will be exposed to bacteria present in the phytosphere. It should be emphasized that, in most cases, the degradation rate of transgenic plant DNA containing ARM genes is expected to be equivalent to that of conventional DNA, and that the natural persistence of plant DNA (including ARM genes) in the environment is not a safety concern beyond the potential uptake of ARM genes by pathogenic bacteria.

Table 2. Studies of the long-term persistence of plant DNA in soil microcosms and in fields.

Source of DNA (genes)	Detection method	Period detected	Reference
Ground tobacco leaf tissues added to soil microcosms (NOS and 35S CaMV)	Extraction of total-DNA, PCR	120 days	Widmer et al., 1996
Tobacco leaves added to soil, potato litter on soil surface (NOS and 35S CaMV)	Extraction of total-DNA, PCR	77-137 days	Widmer et al., 1997
Field sites with transgenic tobacco plants (*aac*I)	Selective plating, extraction of total-DNA, PCR, hybridization	1 year	Paget et al., 1998
Soil microcosms with purified transgenic sugar beet DNA (*npt*II)	Extraction of total-DNA, PCR	3-6 months	Gebhard and Smalla, 1999
Field sites with transgenic sugar beet plants (*npt*II)	Selective plating, extraction of total-DNA, PCR, hybridization	2 years	Gebhard and Smalla, 1999
Stored soil sampled from a potato field, and various other plant species	Natural transformation assay	>2 years	De Vries et al., 2003
Field sites with transgenic sugar beet plants (*npt*II)	Natural transformation assay	1 year	Meier and Wackernagel, 2003

DNA stability in food and feed. Few studies have focused on the stability of DNA in raw or processed food or feedstuff (Pauli et al., 2000, Jonas et al., 2001). Conditions that may cause rapid inactivation or breakdown of extracellular DNA in common food sources are absent according to Bauer et al. (1999). Alexander et al., 2002 investigated the stability of DNA in various canola (*Brassica* sp.) substrates such as whole seed, cracked seed, meal, and diet. In most cases, DNA fragments large enough to contain intact plant genes were detected. However, processing most often decreases the persistence and stability of DNA in food (Pauli et al., 2000). Studies on food processing of e.g. soya, maize, potato and oil seeds, and excessive heat-treatment of plant tissue, such as autoclaving, have demonstrated that such procedures can yield highly fragmented DNA (Chiter et al., 2000, Kharazmi et al 2003a). Using gel-electrophoresis to analyse the degradation of DNA, Chiter et al., (2000) concluded that DNA remains largely intact within feedstuffs such as wet sugar beet pulp, cereal grains, and silage. Two additional studies have also shown variable stability of DNA in silage (Duggan et al., 2000, Einspanier et al. 2001). Methods are continually being developed to detect ARM genes in food and feed (Løvseth et al. 2001), however, DNA degradation during food and feed processing, means that intact ARM genes are only reliably detected in raw materials and unprocessed food.

DNA stability in the digestive tract. Most extracellular DNA present in the digestive system has been demonstrated to undergo substantial degradation by nucleases, produced both by the digestive system and by intestinal bacterial saprophytes. Remaining DNA fragments are excreted in the faeces. Peer-reviewed studies on the persistence of DNA in various digestive systems report variation between organisms and locations (see references cited in Table 1). It is clear that although the majority of DNA is heavily fragmented upon digestion, some DNA fragments of a biologically relevant size (>1 kb) may be excreted in the faeces of various mammals. It should be noted that most studies on DNA stability in the digestive systems of mammals have used purified DNA, whereas most DNA is ingested as complex mixtures in food (Martín-Orúe et al., 2002). In a recent study using GM-soybean meal, Netherwood et al., (2004) reported that whereas some DNA fragments survived passage through the small bowel, transgenes could not be detected in the faeces of human volunteers. Chowdhury et al. (2003a;b) reported that DNA ingested by pigs fed GM-maize was not totally degraded, and that DNA fragments could be detected in caecal and rectal contents. The conditions that permit the persistence of intact DNA fragments during digestion remain undetermined, as does the extent to which such DNA fragments encounter competent bacteria in the digestive tract.

Uptake of ARM genes by bacteria present in the digestive system. The microbial community inhabiting the GIT is characterized by its high population density and wide bacterial diversity (Backhed et al., 2005). Up to 10^{14} bacteria may occur in the GIT, 10 times more than the total number of the somatic and germ cells present in humans. The human small intestine contains relatively low numbers of microbes (10^3 - 10^5/g or ml content) because of low pH and rapid flow in this region. The distal small intestine (ileum) contains higher bacterial numbers (10^8/g or ml content) than the upper part. The large intestine (colon) is the primary site of microbial colonization because of slow turnover and contains large numbers of bacteria (10^{10}-10^{11}/g or ml content), belonging to as many as 400 to 500 different species. The gut bacteria, when combined, may contain >100 times the number of different genes found in our genome. The flow-through of food in the GIT varies between the regions, with the retention of contents in the colon usually for longest (normally 2 to 3 days). The limited retention time provides a constraint on the possibility

of DNA transfer from food from occurring. The concentration of GM-DNA will always be extremely low compared to the total daily intake of DNA present in food, and additionally the DNA from bacteria in the GIT will also "dilute" the intake of GM-DNA.
The animal digestive tract is hypothesized to be an environmental "hot spot" for bacterial gene transfer due to the high concentrations of nutrients and bacteria (Salyers, 1993). Nevertheless, few peer-reviewed studies are available on gene transfer processes and pathways in the digestive system of animals (Mercer et al., 1999b, Nielsen and Townsend, 2004). Currently only a few bacterial species from the digestive system of higher animals have been found to express competence *in vitro,* and none have been found to express competence *in situ* in the colon. Some studies have reported natural transformation to occur *in situ* in oral *Streptococcus* species (Westergren and Emilson 1983, Mercer et al. 1999a, Li et al., 2001). Other bacterial species inhabiting the animal digestive tracts like *Helicobacter pylori* and *Campylobacter* spp. are known to develop competence *in vitro*, although the biological significance of their possible ability to horizontally acquire naked DNA in the digestive system remains unclear. The ability of the human pathogen *H. pylori* to acquire naked DNA is of relevance to our assessment since ampicillin (to which resistance may be conferred by ARM gene $bla_{TEM\text{-}1}$) may be used for the clinical treatment of *H. pylori* infections. Moreover, the bacterium cause infections in the stomach, which is a site where a higher proportion of intact feed-derived DNA fragments is likely to be present, as compared to subsequent sites in the digestive tract. Although stomach acid is thought to be deleterious to the survival of DNA, as inferred from experiments using simulated and natural gastrointestinal fluids (Redenbaugh et al., 1993, Duggan et al., 2000), the many recent observations of feed-derived DNA present in various parts of the digestive system of various animals (see Table 1) show that acid depurination of DNA is not as efficient as previously assumed. With the exception of DNA in saliva (e.g. Duggan et al., 2000), we are not aware of any experimental studies that have identified natural transformation of bacteria to occur in the mammalian digestive tracts. In general, only a few enterobacteria are known to be naturally transformable.

Uptake of DNA by bacteria present in food. Many species of bacteria pathogenic to humans are food contaminants (food-borne pathogens) and grow well in various food sources. Thus, HGT of ARM genes in food is a plausible scenario and there is some evidence that bacteria have the ability to take up naked DNA present in food. Brautigam et al. (1997) and Zenz et al. (1998) detected natural transformation of *B. subtilis*, a common food contaminant, in milk. Bauer et al. (1999) detected plasmid transfer by natural transformation of *E. coli* in various foods, including milk, soy drink, tomato juice, carrot and other vegetable juice, supernatants of canned cabbage, soy beans, shrimps, and various mixes of canned vegetables. Kruse and Sørum (1994) showed that multi-resistance plasmids could readily be transferred by conjugation between bacteria from animals, fish and humans in simulated natural situations such as on a towel contaminated with milk from a cow with mastitis and on a chopping board contaminated with raw salmon. Although limited in numbers and scope, the above studies indicate that unprocessed or processed food may provide bacterial growth conditions suitable for HGT and possibly, natural transformation to occur. However, no studies are available on natural transformation in processed food where the DNA has been naturally present in the food (e.g. food containing ARM genes) since all the reported experimental designs are based on DNA and bacterial recipients being added into the food.

Several studies, as listed previously, demonstrate the persistence and transferability of naked DNA fragments in environmental settings of relevance to the introduction of GMOs. Although the majority of these studies have been performed with high concentrations of both introduced DNA and bacterial recipients, the *ad hoc* group is of the opinion that rare bacterial acquisitions of ARM genes cannot be excluded from occurring in these environments. Further studies are necessary to identify the conditions, sites and factors preventing or promoting such gene transfer events, particularly in the GIT of mammals. Very little information is available on the factors governing and preventing natural transformation of intestinal bacteria.

3.3.2 Experimental studies of horizontal transfer of ARM genes from GMOs to bacteria

Many opinions and assessments have been produced on the potential for ARM genes to be acquired by bacteria (e.g. Nap et al., 1992, FDA 1998, Kok et al., 1994, EFSA, 2004, Van den Eede et al, 2004). Two approaches have been used to elucidate the likelihood of transfer of ARM genes into bacteria. These include experimental laboratory studies on monocultures of bacteria exposed to GMP material (section 3.3.3.1), and examination of bacterial communities exposed to GMP material under semi-natural or natural conditions (section 3.3.3.2). For further reviews on HGT of transgenes in bacteria, see (Nielsen et al., 1998, 2001, Dröge et al., 1998, 1999, Bertolla and Simonet, 1999, Nielsen, 2003).

3.3.2.1 Laboratory studies on bacteria exposed to ARM genes or GMP material

Several laboratory studies have examined the likelihood of ARM gene transfer to defined bacterial species (Table 3). These studies have examined potential bacterial acquisitions of ARM genes, or gene fragments present as either purified DNA or in plant cells. The conditions for uptake of DNA in the monocultures of bacteria are often optimised, thus, presumably, but not necessarily, maximizing the likelihood of ARM gene uptake. The experimental set-up in the laboratory studies allows the potential transfer of ARM genes to be studied in a bacterial population of 10^7-10^9 bacteria, usually grown over a limited time period (<48 h). The limit of detection is often less than 1 successful ARM gene acquisition detected per 10^{10} to 10^{11} bacteria exposed. To date, none of these studies exposing wild-type bacteria to ARM genes have been able to demonstrate stable uptake in the bacterial species examined and therefore indicate that the transfer rates of ARM genes into the bacterial species and strains examined are nonexistent or below the limit of detection.

Many studies on the requirements of integration of species-foreign DNA in bacteria have identified DNA sequence divergence as a main barrier. To increase the likelihood of transgene integration, several of the recent studies on the potential for transfer of ARM genes into bacteria have therefore introduced DNA sequence similarity to the plant transgene in the bacterium. Based on the presence of such defined DNA similarity in the bacteria, several recent studies now show that some bacterial species are capable of accessing and incorporating purified ARM gene fragments *in vitro* and in sterile soil (see references listed in Table 3).
In situ uptake of ARM genes localized in organelles during bacterial colonization of tobacco plants has also been reported when high DNA sequence similarity is present (Kay et al., 2002).

The DNA similarity-based studies produced transformation frequencies of about 10^{-9} to 10^{-6} transformants per exposed bacterium within a 24 h time period.
The studies described in Table 3 indicate that bacteria are physiologically able to take up ARM genes and emphasize the importance of sequence similarity between plant transgenes and the bacterial genome for facilitating detectable levels of transfer. A study by Bensasson et al., 2004, identifies a range of bacteria that harbour DNA sequence similarity to the commonly used pUC18 vector; suggesting sequence similarity conducive to recombination can be found between transgene constructs and bacteria naturally-present in various environments. The possible presence of sequences flanking ARM genes with high DNA similarity to bacteria may thus be of importance for understanding their transfer potential to naturally-occurring bacteria. The recent EFSA guidance document for the risk assessment of genetically plants and derived food and feed, specifically acknowledges this concern in section III.D.6, and recommends that the presence of bacterial sequences within the GMP insert DNA is minimised (EFSA, 2004).

Table 3. Experimental studies on ARM gene transfer from GMPs to bacteria with inserted DNA sequence similarity to the ARM gene.

Plant material	Recipient bacterium	Transfer detected[a]
Purified potato and sugar beet DNA (Gebhard and Smalla, 1998)	*Acinetobacter* sp.	yes
Purified potato, sugar beet and oilseed rape DNA (De Vries and Wackernagel, 1998)	*Acinetobacter* sp.	yes
Purified sugar beet DNA in sterile soil microcosms (Nielsen et al., 2000)	*Acinetobacter* sp.	yes
Purified DNA and infected plants of potato and tomato (Bertolla et al., 2000)	*Ralstonia solanacearum*	no
Purified potato DNA (De Vries et al., 2001)	*Acinetobacter* sp. *Pseudomonas stutzeri*	yes
Infected potato plants (Kay et al., 2002)	*Acinetobacter* sp.	yes
Plant material of *Arabidopsis* (Tepfer et al., 2003)	*Bacillus subtilis*	yes
Purified tobacco DNA (Ceccherini et al., 2003)	*Acinetobacter* sp.	yes
Purified DNA of sugarbeet (Meier and Wackernagel, 2003)	*Acinetobacter* sp.	yes
Purified DNA of potato, *in vitro*, in food and gnotobiotic mice (Kharazmi et al., 2003b)	*Streptococcus gordonnii*	yes

[a]Transfer frequencies range between 10^{-9} to 10^{-4} transformants per exposed recipient, typically over a 24 to 48 h transformation period.

3.3.2.2 Examination of bacterial communities exposed to ARM genes or GMPs under natural conditions

Four published studies have investigated whether transgenes (e.g. ARM genes or epsps genes) present in GMPs can spread horizontally to exposed microbial communities in agricultural soils and in the intestines of human volunteers (Paget et al., 1998, Gebhard and Smalla, 1999, Badosa et al., 2004, Netherwood et al., 2004). The experimental designs of these studies are based on the phenotypic screening of bacterial populations for putative transformants carrying the monitored genes. The investigations also sampled the non-culturable fraction of bacteria present in soil or the intestine without identifying HGT events. Horizontal gene acquisitions occurring into non-culturable bacteria (generally non-culturable, or not responsive to the media and conditions selected in the studies) remain exceedingly difficult to detect (Nielsen and Townsend, 2004). None of the monitoring studies conclusively detected transgene acquisitions in the genomes of the exposed bacterial populations. It should be emphasized that the approach used in these studies provides only a limited ability to detect rare bacterial transformants carrying the transgene. The probability that the analysis will reveal transformants is exceedingly low due to the high number of bacteria naturally present in the environments sampled, and also the high intrinsic background resistance to the antibiotics used to select bacteria carrying ARM genes. It has been estimated that all the field studies to date have examined HGT processes occurring in bacteria present in less than 2 g of combined sample material (Nielsen and Townsend, 2004). The sampling time has also been questioned in these studies, since it may take a prolonged period (weeks, years, or decades), for the transformants carrying ARM genes to reproduce to sufficient numbers to be detected by monitoring (Nielsen and Townsend, 2004).

The many studies described above demonstrate that horizontal transfer of ARM genes can occur under highly optimised conditions and that no inherent barrier is present to prevent the process from occurring. Sampling limitations prevent HGT monitoring strategies from being efficiently applied to natural bacterial communities exposed to ARM genes.
The *ad hoc* group is of the opinion that current studies demonstrate the potential for horizontal acquisitions of ARM genes in bacterial communities, and that further studies are necessary to determine the relevance of these experimental studies to natural GMO conditions. Further, that advances in sampling strategies and bacterial population genetics are necessary to enable informative monitoring of HGT processes occurring in natural bacterial populations exposed to ARM genes from GMOs.

Table 4. Studies performed to examine horizontal transfer of ARM or *epsps* genes into soil or gut bacteria.

Transgenic plants	Methods of detection	Transfer detected	Reference
Tobacco plants grown in field sites in France	Cultivation, DNA extraction, hybridization and PCR	No	Paget et al., 1998
Sugar beet plants grown in field sites in Germany	Cultivation, DNA extraction, hybridization and PCR	No	Gebhard and Smalla, 1999
Corn plants grown in field sites in Spain	Cultivation, DNA extraction, hybridization and PCR	No	Badosa et al., 2004
Human volunteers consuming soy bean meal	Cultivation, DNA extraction, hybridization and PCR	Not during the investigations, although indications were found that transfer had occurred prior to the start of the study	Netherwood et al., 2004

3.3.3. Resistance mechanisms and the prevalence of ARM gene homologues in bacterial communities

The assumption that there is a low risk of increased resistance development associated with using particular ARM genes has been based on the observed high prevalence of resistance traits in environmental bacteria (Kelch and Lee, 1978, Henschke and Schmidt, 1990). However, it is necessary to determine the genetic basis for the observed resistance in order to make these studies informative for biological risk assessment. Only a few peer-reviewed studies are available on the concentrations and the locations of resistance gene homologues to ARM genes in non-clinical environments (e.g. Leff et al., 1993, Smalla et al., 1993, 1997, van Elsas and Smalla, 1995, Aarestrup et al., 2000, Sandvang and Aarestrup, 2000, Seveno et al., 2002). In clinical settings, AR in bacteria are most often monitored and described by phenotype (NORM/NORMVET 2004) (see also Appendix). Therefore in most cases it is difficult to link an observed phenotypic resistance trait in a clinical isolate specifically with the occurrence of a particular AR gene.

3.3.3.1. Aminoglycosides, resistance mechanisms and the prevalence of resistance genes

Aminoglycosides constitute a large group of hydrophilic antibiotics, which include; amikacin, gentamicin, hygromycin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin, and

tobramycin. These chemical compounds are products of actinomycetes (soil bacteria) or semi-synthetic derivates of the natural products. Aminoglycosides are characterized by the presence of an aminocyclitol ring linked to amino-sugars in their structure. They are active against aerobic and facultative aerobic gram-negative bacteria and some gram-positive bacteria like staphylococci. Aminoglycosides are valuable antibiotics for the treatment and prophylaxis of various infections (Vakulenko and Mobashery, 2003). In Norway, some aminoglycosides are considered very important drugs in the empirical treatment of systemic bacterial infections, due to their broad-spectrum bactericidal effect and the low prevalence of aminoglycoside-resistance (NORM/NORM-VET 2004).

Aminoglycoside resistance mechanisms. Aminoglycosides exert their effect by interfering with translational fidelity during protein synthesis, by interacting with the decoding region of the A-site rRNA on the 16S domain of the ribosome (Moazed and Noller 1987). There are three known mechanisms of resistance against aminoglycosides; i) decreased intracellular concentration of the drug; ii) target modification; and iii) enzymatic drug modification (Magnet and Blanchard 2005). Transport defects or membrane impermeability have been reported in some aminoglycoside resistant strains of *Pseudomonas aeruginosa* and other gram-negative bacteria (Mingeot-Leclercq et al., 1999). Resistance to streptomycin can occur by target modification since the agent binds to a single site on the 30S subunit of the ribosome. This mechanism is not recognised as the reason for resistance to other aminoglycosides, since they bind to multiple sites on both ribosomal subunits, and therefore high-level resistance would not be selected by a single mutational step (Kucers et al., 1997). The most clinically important resistance mechanism is enzymatic drug modification. The genes encoding aminoglycoside-modifying enzymes can often be found on, and disseminated by, plasmids and transposons. Aminoglycoside-modifying enzymes catalyse the covalent-modification of specific amino or hydroxyl functions, resulting in a chemically modified drug that binds poorly to ribosomes and for which the energy dependent process (phase II) of accelerated drug uptake also fails to occur. This may frequently lead to high-level resistance (Davies and Wright 1997). More than 50 different aminoglycoside modifying enzymes have been identified, which are classified into three major classes (AAC, ANT, APH) according to the type of modification (Shaw et al., 1993). These genes are often found on mobile genetic elements such as transposons and plasmids (Derbise et al., 1997, Gibreel et al., 2004). It has been suggested that the enzymes are derived from microorganisms that make the aminoglycoside, or from the mutation of genes that encode enzymes involved in cellular respiration (Gilbert, 2000). The specific names of the enzymes discussed in relation to ARM genes are HPT = APH(4)-F (hygromycin resistance), NPTII = (APH3`)IIa (kanamycin resistance) and STR = ANT(3``)1a (streptomycin resistance).

Prevalence of the *npt*II resistance gene (Group 1). Few studies are available to provide accurate information on the environmental distribution and copy numbers of the *npt*II gene in Europe. Most of the available peer-reviewed studies suggest a very low prevalence of *npt*II genes among bacteria in non-clinical environments (Leff et al., 1993, Smalla et al., 1993). The Leff et al. (1993) study was conducted in the USA. The only environmental study of *npt*II distribution in Europe, of which we are aware, was performed on a limited sample set from the Netherlands and Germany (Smalla et al., 1993, Gebhard and Smalla, 1999). In this study the *npt*II gene was not detected in soil, but was found in sewage and manure samples taken in the Netherlands and Germany. The *npt*II gene has been described as being rarely found in clinical isolates (Shaw et

al., 1993). We are not aware of any peer-reviewed studies that have described the environmental prevalence of *npt*II genes in Scandinavia. Unpublished studies (K. G. Berdal, pers. comm.). of 60 imported feed samples tested in Norway by the National Veterinary Institute (routine surveillance requested by public authorities) in 2003 and 2004, 5% gave positive PCR amplification of *npt*II gene fragments and all the *npt*II fragment positive samples were also positive for several other antibiotic resistance genes by PCR analysis

Knowledge of the level of phenotypic kanamycin resistance can provide some insight into the overall occurrence of resistance, although the genetic basis for the resistance trait remains unknown. In soil, phenotypic kanamycin resistance is widespread, with approx 10^5 resistant bacteria per gram of soil (Henschke and Schmidt, 1990, Smalla et al., 1993, Nielsen et al., 2000b). In the USA, a low level of phenotypic kanamycin resistance has been reported in poultry litter (Kelley et al., 1998), and fresh water samples (Kelch and Lee, 1978). A study of the antibiotic resistance profiles of *Campylobacter jejuni* from wild birds in Sweden did not detect any isolates resistant to neomycin among 274 isolates (Waldenström et al., 2005), thus suggesting a low prevalence of the *npt*II gene in wild birds that are can be a reservoir of these enteropathogenic bacteria.

In clinical settings, the highly variable usage of aminoglycosides results in variable degrees of phenotypic observable resistance between clinical strains, hospitals, and countries (Vakulenko and Mobashery, 2003) In the USA, the SENTRY surveillance programme reported that the 4 most common gram-negative bacteria in bloodstream infections were susceptible to the aminoglycosides tested (Pfaller et al., 1998). Similarly, low levels of phenotypic resistance were found in European clinical isolates (Schmitz et al. 1999). A small increase in the aminoglycoside resistance patterns has been observed (see Vakulenko and Mobashery, 2003). A low prevalence of aminoglycoside resistance determinants mediating isolated resistance to kanamycin in Norwegian blood culture isolates of *Staphylococcus epidermidis* has been reported, suggesting a very low prevalence of the *npt*II gene among human clinical isolates in Norway (Klingenberg et al., 2004). The phenotypic resistance situation to kanamycin in Norway remains very low as documented in yearly surveillance reports (SNT Report 1998, 1999, Kruse 1999, 2000, Kruse and Schau 2001, Kruse and Skov Simonsen 2001, NORM/NORM-VET 2001, 2004) suggesting an even lower prevalence of the *npt*II gene.

Although the prevalence of pathogenic and environmental bacterial isolates resistant to kanamycin in Norway is low, the data available suggests *npt*II gene copies are present in Norwegian environments and in feed sources. The data available does not facilitate precise estimates to be made of the copy numbers of the *npt*II gene in Norwegian environments.

Prevalence of the *hpt* resistance gene (Group 1). We are not aware of any studies that have examined the environmental or clinical occurrence of the *hpt* gene.

Prevalence of the *str* resistance gene (Group 2). Only a few studies are available on the environmental distribution of the *str (ant*(3``)1a) gene in Europe. One comprehensive study that sampled soil, the rhizosphere, manure, activated sludge and seawater from several European countries reported the presence of the *ant*(3``) gene in all the habitats examined, suggesting a widespread occurrence of this gene in various bacterial species and environments (van Overbeek

et al., 2002). The study of *Campylobacter jejuni* from wild birds in Sweden found no streptomycin resistant isolates among the 274 isolates examined (Waldenström et al., 2005), suggesting a low prevalence of the *ant*(3``)1a gene in these enteropathogenic bacteria. It is, however, difficult to estimate the total copy number of *ant*(3``)1 genes present in the various environments studied. We are not aware of any comprehensive studies on the prevalence of the various *str* genes in Norway. Thus, the occurrence in various environments and the bacterial species distribution of the *ant*(3``)1a gene in Norway is unknown, and it is possible that the situation in Norway may differ from that in other European countries due to variations in streptomycin usage. Nevertheless, as streptomycin has been commonly used in veterinary medicine in Norway for many years and as phenotypic streptomycin resistance is relatively frequently observed (NORM/NORM-VET, 2004), it is possible that the environmental distribution of the *str* gene in Norway is similar to the situation in Europe.

3.3.3.2. Ampicillin, resistance mechanisms and the prevalence of resistance genes

Ampicillin is an aminopenicillin, belonging to the family of β-lactam antibiotics. β-lactam antibiotics are one of the largest groups of commercially available antibiotics and include the penicillins and the cephalosporins, which have related structures and similar mechanisms of action. Ampicillin is considered a broad-spectrum penicillin and inhibits a set of transpeptidases that catalyse the final cross-linking reaction of peptidoglycan synthesis in bacteria. The loss of the stability conferred by the wall eventually leads to cell lysis. Ampicillin has been extensively used to treat bacterial infections including shigellosis (dysentery), gonorrhoea, meningitis, and streptococcal and staphylococcal infections since 1961. However, the increased prevalence of various betalactamase genes in human pathogens has significantly limited its clinical use to respiratory and urinary tract infections caused by betalactamase-negative, ampicillin susceptible bacteria confirmed by antimicrobial susceptibility analysis (see below).

Ampicillin resistance mechanisms. High-level bacterial resistance to the β-lactams is primarily due to the hydrolysis of the antibiotic by a β-lactamase enzyme (Bush et al., 1995). A wide diversity of ampicillin resistance genes encoding β-lactamases has been described that belongs to various families such as TEM, SHV and CTX. TEM-1 is usually a plasmid encoded β-lactamase, commonly encountered in *E. coli* that inactivates narrow-spectrum cephalosporins, cephamandole, and cephoperazone, and all gram-negative active penicillins except temocillin. Importantly, many extended-spectrum β-lactamases (capable of hydrolysing extended spectrum cephalosporins) derive from simple point mutations in the TEM-1 allele. More than 140 such amino acid substitution variants of the TEM-1 allele have been described. The *bla* gene encoding TEM-1 β-lactamase is widely used as a selective marker conferring ampicillin-resistance in molecular biology studies and is present in the frequently-used pBR and pUC plasmids. Some of the pUC cloning plasmids have also been inserted into plants, for instance in the development of GM insect-resistant maize, including event *Bt*176 (Malik and Saroha 1999).

Prevalence of the $bla_{TEM\text{-}1}$ resistance gene (Group 2). The environmental prevalence of $bla_{TEM\text{-}1}$ alleles is presently mostly unexplored. Unpublished studies suggest that their prevalence may be very low, and is below the limit of detection in the intestines of seals, polar bears, and in soil samples taken from Arctic regions (T. Glad, K. M. Nielsen, unpublished). Similar low levels

have been noted in various agricultural soil samples from New Zealand (P. Myren, P. Carter, K. M. Nielsen, unpublished). However, PCR positive signals for *bla*$_{TEM}$ genes have been detected in a commercially sold compost soil in Italy (L. Brusetti, D. Daffonchio, K. M. Nielsen, unpublished).

The clinical prevalence of *bla*$_{TEM\text{-}1}$ is likely to vary between clinical strains, hospitals, and countries. Most ampicillin-resistant (Amp^R) clinical isolates of *E. coli* harbor the TEM-1 allele (Livermore, 1995, and Sundsfjord A, unpublished). The prevalence of ampicillin-resistance in blood culture isolates of *E. coli* in various European countries ranges between 25 and 65 % (EARSS, 2002). This level of ampicillin-resistance also reflects the prevalence of Amp^R in uropahogenic *E. coli*, thus limiting the usefulness of ampicillin in the empirical treatment of urinary tract infections as well as in septicaemia. The prevalence of resistance in clinical strains is consistent with the observed occurrence of 23 % of faecal *E. coli* strains from healthy humans in Denmark demonstrating ampicillin-resistance (DANMAP 2004). Clinical studies of *E. coli* and other enterobacterial isolates suggest that the *bla*$_{TEM\text{-}1}$ gene is present in >20 % of isolates from the intestinal system of humans in Norway (A. Sundsfjord, pers. comm.). The emergence and spread of β-lactamases within *Staphylococcus aureus* and *E. coli*, the two most prevalent bacterial species in blood culture specimens, has significantly reduced the clinical usefulness of ampicillin during the last 10 years. The *bla*$_{TEM\text{-}1}$ gene seems to be the major determinant encoding ampicillin-resistance in *E. coli*, whereas the *blaZ* gene dominates among staphylococci. However, a leading expert on *bla*$_{TEM}$ genes Prof. P. Courvalin states that although the *bla*$_{TEM\text{-}1}$ allele is prevalent in *E. coli,* it is currently not ubiquitous amongst commonly encountered bacteria that are pathogenic to man; i.e. streptococci, enterococci, *Neisseria* spp., and *Haemophilus influenzae* (Courvalin, 1998). As a consequence, antibiotics to which *bla*$_{TEM\text{-}1}$ confers resistance are still among the most sold antibiotics in the world. Therefore, whereas the *bla*$_{TEM\text{-}1}$ gene may be prevalent in some previously susceptible species, the current geographical occurrence and species distribution of resistance is not hindering the continued clinical use of ampicillin, and related derivatives, for treatment of infections. The Norwegian surveillance system for antibiotic resistance in human medicine (NORM) have shown that *bla*$_{TEM}$-mediated ampicillin resistance is encountered in 30 % and 6 % of clinical isolates of *E. coli* and *H. influenzae*, respectively (NORM/NORM-VET 2001, 2003). The current prevalence of *bla*$_{TEM}$-mediated ampicillin resistance in *Neisseria gonorrhoea* is not available. In 2002, 3% of 120 *E. coli* isolates from faecal samples from 120 cattle were ampicillin resistant, whereas only 0.8% of 118 *E. coli* isolates from faecal samples from 118 sheep were ampicillin resistant (NORM/NORM-VET 2001, 2003). Ampicillin is sometimes used therapeutically in cattle and sheep in Norway. Of the sheep included in this study, 70% were lambs slaughtered at about 6 months of age. Lamb production in Norway is extensive, and the lambs spend a large part of their lives roaming freely on rough, upland grazing. Another survey in 2002 showed that none of 42 *E. coli* isolates from faecal samples from 42 reindeer in Norway were ampicillin resistant. These data further demonstrate a low environmental occurrence of ampicillin-resistance in Norway.

3.3.3.3. Chloramphenicol, resistance mechanisms and the prevalence of resistance genes

Chloramphenicol was originally isolated from *Streptomyces venezuelae* and is now synthetically-produced. The compound mainly exerts a bacteriostatic effect on a wide range of gram-positive

and gram-negative organisms (Schwarz et al., 2004). In addition, it is active against *Rickettsia*, *Chlamydia* (psittacosis-lymphogranuloma), and *Mycoplasma* (Shaw, 1983). Chloramphenicol is now very seldom used for systemic treatment of bacterial infections, however, it is often used for the empirical topical treatment of bacterial conjunctivitis. Chloramphenicol acts by binding to the 50S ribosomal subunit and blocking the formation of the peptide bond by inhibiting peptidyl transferase activity and inhibiting protein synthesis (Schlunzen et al., 2001)**.** It is a potent inhibitor of mitochondrial protein synthesis in eukaryotic cells.

Chloramphenicol resistance mechanisms. Resistance against chloramphenicol in bacteria is mainly due to enzymatic inactivation. Resistance to chloramphenicol is conferred by the *cat* gene, which encodes for the enzyme chloramphenicol acetyltransferase (Murray and Shaw 1997). There are also reports of other mechanisms of chloramphenicol resistance, such as efflux systems, inactivation by phosphotransferases, mutation of target sites and permeability barriers (Shaw et al., 1993).

Prevalence of the *cat* resistance gene (Group 2). We are not aware of studies that have examined the clinical or environmental prevalence of the *cat* gene in Norway or Europe. The study of the antibiotic resistance profiles of *Campylobacter jejuni* from wild birds in Sweden found no chloramphenicol resistant isolates among the 274 isolates (Waldenström et al., 2005), suggesting a low prevalence of the *cat* gene in these enteropathogenic bacteria. In Norway in 2002, 0.8% of 120 *E. coli* isolates from faecal samples from 120 cattle were chloramphenicol resistant, whereas none of 118 *E. coli* isolates from faecal samples from 118 sheep were chloramphenicol resistant (NORM/NORM-VET 2001, 2003). A survey conducted in Norway in 2002, showed that none of 42 *E. coli* isolates from faecal samples from 42 reindeer were chloramhenicol resistant (NORM/NORM-VET, 2002). These data demonstrate a low environmental occurrence of chloramhenicol-resistance in Norway. It should be noted that chloramphenicol use is prohibited for systemic treatment of food-producing animals in Norway.

3.3.4 Identification of selective conditions favouring bacteria harbouring ARM genes

In order for rare bacterial transformants, carrying acquired ARM genes, to outnumber and establish in a larger bacterial population, positive selection is usually required (Nielsen and Townsend, 2001, 2004). It should be noted that today's clinical problem of AR is most often not the direct result of high transfer frequencies of resistance genes in clinical environments and, hence, high numbers of first generation of clinically troublesome resistant bacteria. More often, the resistance problems only arise after the selective amplification of bacterial populations harbouring the acquired resistance determinant, which have descended from the original bacterial cell that acquired the trait. Identification of those selective conditions and the quantification of the growth dynamics of bacteria harbouring ARM genes are thus required in order to understand the potential for rapid amplification of bacteria carrying newly-acquired resistance traits. Unfortunately, our current understanding of selection in complex natural environments is limited; the data are sporadic and quantification is exceedingly difficult (Recorbet et al., 1992, van Elsas, 1992, Wellington et al., 1993, Oliveira et al., 1995). Nevertheless, information on the distribution and usage patterns of pharmaceutically-produced antibiotics allows the identification of

environments where strong positive selection of resistance carrying bacteria is likely to occur. The annual NORM/NORM-VET report (NORM/NORM-VET, 2004) provides an overview of the current usage of antibiotics in Norway. As seen in Table 5, in Norway neomycin, ampicillin, streptomycin and chloramphenicol are used in human medicine, and neomycin, streptomycin, and penicillin are used in veterinary medicine. Thus, with the exception of hygromycin, all the ARM genes categorised as belonging to either Group 1 or Group 2 by the EFSA opinion (EFSA, 2004) encode resistance to compounds used therapeutically in Norway. An increase in the resistance level to these antibiotics will therefore necessarily have an effect on antibiotic usage patterns and infection treatment efficiency.

Table 5. Total sales of relevant antibiotics for human and veterinary medicine in 2004 in Norway (in kg of active substance)[A].

Antibiotics	Human	Veterinary
Group 1		
Neomycin	0,01	31
Group 2		
Benzylpenicillin	?	1991
Ampicillin	320	-
Amoxicillin	?	202
Streptomycin	0,6	131
Streptomycin in combination with penicillin	_	1365
Chloramphenicol	30	-[B]

[A](NORM-NORMVET, 2004), excluding aquaculture.
[B]111 kg of florfenicol was used therapeutically for farmed fish.

The *ad hoc* group is not aware of comparable comprehensive data sets on the use of these antibiotics in the EU. Unfortunately, data on the use of antibiotics are not generally available for most EU countries and large variation in non-hospital usage of antibiotics across Europe, and in the classes of antibiotics used, has also been reported (Cars et al., 2001). This information reveals that there are clear knowledge gaps on the actual European usage of antibiotics relevant to the ARM genes that have been classified into Groups 1 and 2. It can, however, be inferred from the Cars et al. (2001) study, that most European countries have an overall higher usage than Norway of the specific ARM gene relevant antibiotics as listed in Table 5. For instance, Nap et al. (1992) estimate that 30 tons of neomycin and kanamycin is used annually in Dutch agriculture. The DANMAP report (DANMAP, 2004) shows that 11 675 kg of aminoglycosides, 317 kg of amphenicols, 20 950 kg of β-lactamase sensitive penicillins are used annually for veterinary purposes in Denmark. The SVARM report (SVARM 2004) quantifies the annual Swedish use of antimicrobial drugs for veterinary purposes as penicillins and aminopenicillins 8689 kg, and aminoglycosides 606 kg. Interestingly, the review by Nap et al. (1992) provides calculations that do not exclude the possibility that the concentration of these aminoglycoside antibiotics may reach selective levels beyond their intended destination, e.g. in soil. Thus, selective conditions for

ARM gene carrying bacteria can possibly be found beyond clinical and veterinary treatment settings. Halling-Sørensen et al. (1998) provide an extensive review of pharmaceutical substances found in various environments.

The *ad hoc* group considers that given the documented usage of antibiotics in Norway, the positive selection of bacteria carrying ARM genes (and naturally-occurring gene homologues) may occur under certain conditions. This potential amplification of ARM genes in bacteria must be seen in relation to the exposure (levels and locations) of bacteria to naturally-occurring ARM gene homologues under the same conditions.

3.3.5. Conclusions from section 3.3

Based on the available data from experimental studies, horizontal transfer of ARM genes is likely to be an infrequent event and relies on the presence of stretches of similarity between the transferred DNA (containing the ARM gene) and the DNA of the bacterial recipient. No clear quantitative value exists to relate relevant frequencies to potential environmental impact. Thus, frequencies are of little predictive value in our assessment, particularly since the relevant frequencies may be well below that which can be measured in the laboratory or the field (Heinemann et al., 2004, Nielsen and Townsend, 2004). The impact of any rare bacterial acquisitions of ARM genes must be considered in relation to the potential for those bacterial transformants to be amplified selectively in the larger bacterial population. Moreover, the HGT rate of ARM genes must be considered in relation to the exposure rate and HGT rate to bacteria of naturally occurring homologues to the ARM genes. Addressing the range, quality and consensus between studies forming the basis of our knowledge of natural reservoirs of ARM gene homologues, and the knowledge of ARM gene selective conditions, is therefore essential in our assessment.

Group 1. For the Group 1 gene *npt*II, the *ad hoc* group considers that available data suggests the occurrence of this gene in pathogenic bacteria in Norway to be low. Veterinary usage of aminoglycosides such as neomycin in Europe, including Norway, may create selective conditions for bacterial transformants harbouring ARM genes. The *npt*II genes present in GMOs are generally regarded as not being a significant source of AR genes in comparison to the *npt*II genes already present in bacterial communities. However, in the absence of a quantitative understanding of the copy numbers of ARM genes naturally present in antibiotic-exposed environments, it is difficult to estimate the relative contribution of ARM genes to the total copy number of homologous AR genes in the environment. Further information on the natural occurrence of the *npt*II gene (i.e. relative copy numbers) in relevant Norwegian environments is necessary to provide a quantitative assessment of the possible effects of introducing the nptII gene as an ARM to Norway. Little information is available on the distribution and ecology of the *hpt* gene. However, due to the limited clinical and veterinary importance of this gene, the *ad hoc* group has not identified specific concerns on the use of this gene as an ARM.

Group 2. The $bla_{TEM\text{-}1}$ gene and the *str* gene appear to have a broad distribution in clinical settings throughout Europe, including Norway. However, the species and environmental distribution, and animal reservoirs, for the $bla_{TEM\text{-}1}$ gene are limited. Arguably, the additional

introduction of these genes in an ARM context may provide only a limited contribution to the overall gene prevalence (copy numbers) of these genes in humans in Norway. Less information is available on the *cat* gene distribution. The antibiotics to which the $bla_{TEM\text{-}1}$ gene and the *str* gene confer resistance are widely used for the treatment of infections in clinical and veterinary medicine in Norway (NORM/NORM-VET, 2004). The broad usage and utility of these antibiotics suggests caution should be applied in the dissemination of the corresponding resistance genes in environments that are selective for bacterial transformants carrying ARM genes.

The *ad hoc* group finds that the lack of quantitative data on the copy numbers of naturally-occurring ARM gene homologues present in various environments makes it difficult to assess the relative copy numbers contributed by introduced GMOs harbouring ARM genes.

4. Overall assessment

ARM genes are originally isolated from naturally occurring bacteria present in species-diverse microbial communities. Thus, microorganisms in environmental, commensal and clinical environments are naturally exposed to such resistance genes at variable levels. Moreover, bacteria are known to transfer AR genes frequently between cells and species (Derbise et al., 1997, Gibreel et al., 2004). AR genes are known to occupy a range of genomic locations in various microorganisms making them some of the most dynamic genetic entities known. Mammalian exposure to ARM genes should be seen in relation to the daily exposure to a huge diversity of genes that mammals normally experience, both from ingested food, and also from intestinal bacteria.

The large-scale release of GMOs containing ARM genes may thus not necessarily introduce new AR genes into a particular environment. However, the GMO introduction may change the environmental persistence and concentrations, of resistance genes and alter the dynamics of their exposure to various microorganisms. Moreover, the insertion of ARM genes into eukaryotic chromosomes in GMOs will necessarily alter the genomic locations of ARM genes substantially from those locations AR genes usually occupy in prokaryotic genomes. The changed genomic insertion sites and regulatory sequences may produce novel ARM gene expression patterns, epigenetic effects and may introduce post-translational modifications of the active enzymes. The effects of such changes, some of which are only hypothetical, are addressed in the risk assessment of GMOs intended for commercial use in Europe (EFSA, 2004).
Based on the available information, the *ad hoc* group has reached the following assessment:

Potential direct effects of ARM proteins and genes on mammals. There is a only a limited number of publicly available peer-reviewed experimental studies on the potential toxic, allergenic or other effects on mammalian cells of ARM-encoded proteins or ARM genes. The conclusions advocated here of a low risk produced by mammalian exposure to ARM gene encoded enzymes or ARM genes are mainly based on inference from the lack of observable effects from human exposure to naturally occurring counterparts of the ARM gene encoded proteins or ARM gene homologues. Although the natural concentrations of these resistance-producing enzymes or genes

are often unknown, consequences from natural exposure of mammals to these enzymes or genes have, to the extent investigated, not been reported.
From the published studies on the uptake and tissue distribution of food-derived DNA in several mammalian species, it can be assumed that humans will infrequently take up intact ARM genes present in food from the intestinal tract into the bloodstream. The lack of a detailed understanding of the uptake mechanisms, transport pathways and degradation dynamics of food-derived DNA in the bloodstream of mammals represents a major knowledge gap that warrants further research. Moreover, the lack of quantitative data on DNA fragment size makes precise predictions of exposure rates of intestinal host cells to DNA and the relevant physical locations of DNA impossible. The possible interactions of ARM gene encoded proteins and their enzymatic activity with other proteins (proteome) in mammalian cytoplasms remains unexplored and should be further clarified. There are, however, no indications in the scientific literature suggesting ARM genes will produce negative effects if accidentally present in mammalian cell cytoplasm.

Potential indirect health effects of ARM genes on mammals. Some of the ARM genes in Groups 1 and 2 encode resistance to antibiotics that are used therapeutically in human and veterinary medicine (Kruse and Jansson, 1997, EFSA, 2004). It is possible that pathogenic bacteria exposed to GMOs may infrequently acquire ARM genes that may ultimately lead to a reduction in the number of compounds available to treat bacterial infections in humans, plants and animals. A number of recent experimental studies show that ARM genes can be horizontally acquired by bacteria under optimised conditions (See table 4). Unfortunately, frequencies alone cannot be used to predict the long-term effects of sporadic gene transfer events. The relevance, sites and clinical effects of rare ARM gene transfer events should be seen in relation to the frequencies in which parallel AR gene acquisitions occur in bacteria from gene sources other than GMOs. A hypothetical negative outcome of the large-scale usage of ARM genes in GMOs includes significant alterations in genomic context, concentration levels and exposure routes of antibiotic resistance genes to relevant pathogens, thus increasing their potential to respond to antibiotic exposure by utilizing horizontally acquired genes. Knowledge of the natural exposure rates of bacterial communities to ARM gene homologues and selective compounds is essential in order to understand the comparative risk from ARM gene usage in GMOs. Some data are available on phenotypic and genotypic resistance distributions among selected environmental samples and clinical isolates in various locations across Europe. However, no systematic studies are available that estimate the copy numbers of the different ARM gene homologues in various environments. Without a clear understanding of the ARM gene copy number released in various GMOs, and the copy numbers of the corresponding naturally-occurring AR genes, it is difficult to estimate the relative contribution of ARM genes in GMOs to the overall exposure of bacteria to AR genes. Current understanding of resistance development in pathogenic bacteria does not allow an accurate prediction to be made on the effect of introducing new sources of resistance genes at various concentrations and locations.

As outlined in the EFSA opinion (2004), two criteria can be adopted to approximate and supplement the lack of a direct predictive capability. These two criteria are 1) identification of the current natural reservoirs of resistance genes (section 3.3.3); and 2) identification of selective conditions favouring bacteria acquiring ARM genes (section 3.3.4). These assist in assessing the possible relative contribution of the ARM gene release from GMOs to the overall AR gene reservoir, and determining whether rare bacterial transformants that have acquired ARM genes

are likely to undergo directional selection and amplification leading to clinically significant populations. Unfortunately, it is unclear how the EFSA opinion and assessment quantitatively define the prevalence of the genes in relation to the group categorization and assessments made, and it is also unclear if the prevalence argument is based on considerations of the ARM copy number only, or if the relative presence of ARM gene homologues among relevant clinical isolates in different countries is considered too.

Our literature survey and expert consultation suggests that the Group 1 gene *npt*II is only infrequently found in natural and clinical environments in Norway. Higher levels of the gene have been found in manure and sewage in a limited number of samples from the Netherlands and Germany. However, since the past and present agricultural usage of antibiotics varies considerably between countries, these observations need to be extended to Norwegian environments. Given the low level of phenotypic resistance to neomycin and kanamycin in pathogenic bacteria in Norway, more information is needed on the *npt*II gene prevalence (i.e. relative copy number considerations) in relevant Norwegian environments.
Little information is available on the distribution and ecology of the *hpt* gene. However, due to the limited clinical and veterinary importance of this gene, the *ad hoc* group has not identified any specific concerns on the usage of this gene as an ARM.

The $bla_{TEM\text{-}1}$ allele is unlikely to be present at high concentrations in natural environments in Norway. However, clinical studies of *E. coli* and other enterobacterial isolates suggest that the $bla_{TEM\text{-}1}$ gene is present in considerable quantities in the intestinal system of humans. Despite the apparently high prevalence of this gene among bacteria that are widely distributed in anthropogenic environments, the antibiotics to which the $bla_{TEM\text{-}1}$ gene confers resistance remain some of the most widely used in the world. This suggests the resistance gene has a narrow species distribution and is present within these host bacterial populations at high proportions. Thus, there is no clear link between the high prevalence of resistance genes in defined species and environments, and the potential for rapid dissemination to other sensitive species during clinical ampicillin-treatment.
The *str* gene seems to have a broad distribution among various habitats in Europe, although copy number estimates are rarely provided.
Little information is available on the *cat* gene distribution among species and environments in Europe.

The antibiotics to which the $bla_{TEM\text{-}1}$ gene and the *str* gene confer resistance are widely used in clinical and veterinary treatment of infections in Europe, including Norway (NORM/NORM-VET, 2004). The broad usage and utility of these antibiotics, combined with the observation of emerging ampicillin resistance in previously susceptible species, suggests caution should be applied in the dissemination of resistance genes in environments that are selective for bacterial transformants carrying specific ARM genes.

5. Uncertainty in the assessment and some identified knowledge gaps

Uncertainty and divergence in opinion is a natural part of the scientific process, and is particularly apparent in the application of novel technologies. Most risk assessments for novel technology applications pinpoint knowledge gaps and generate a number of questions, some with no clear scientific answers or public consensus. This is also the case in assessing the risks associated with the introduction of ARM genes as selectable markers in GMOs.

A variety of assumption-based reasoning and information sources have been used in the biological risk assessment of ARM genes to resolve some of the questions raised. The basis for the assumptions made and the information sources include, i) direct test results submitted by the GMO developers; ii) experimental data available in the peer-reviewed literature; iii) published and/or communicated historical and comparative experiences and observations of similar biological systems; and iv) submitted or conducted expert evaluations of the outcomes of conceived worst case scenarios.

The robustness, nature and quality of the assumptions made and the information sources available can lead to contested outcomes of the risk assessment. The scientific strength and, hence, social robustness, of any risk assessment depends on how the uncertainties embedded in the prevailing paradigms are identified, addressed and communicated. The *level* of uncertainties related to the biological risk assessment can be divided into various categories ranging from statistical uncertainty to total ignorance (Strand, 2001, Walker et al., 2003). The overall concept of uncertainty is also defined by its *location* and *nature* (Walker et al., 2003). Uncertainty related to the location includes the problem framing and model context, model use, interpretation and relevant parameters. The nature of the uncertainty relates to the distinction between epistemic uncertainty that can be reduced with more research, and variability uncertainty due to inherent stochasticity within the system that cannot be fully resolved with empirical efforts.
It is important that regulatory bodies recognize, address and transparently communicate how they deal with the uncertainty inherent in the process of biological risk assessment. Regulatory bodies often operate to strict deadlines and must therefore act with the information on hand, since limited time and resources rarely allow extensive experimental investigations to be initiated to support the assumptions made or conclusions drawn. This is also the case for our assessment of the ARM genes.

In the present assessment of the ARM marker genes, the *ad hoc* group notes that most of the assessment is based on comparative experience and inference from the natural presence of ARM gene homologues, rather than on direct experimental or epidemiological verification of the absence of negative effects. The inference from lack of observable effects from human or environmental exposure to natural counterparts of ARM gene homologues forms the baseline for our assumptions of no direct biological effect of ARM genes in humans or the environment. In our assessment of the indirect effects of ARM genes, we identify many areas with clear uncertainty that relates to the location (HGT context, HGT model application, HGT parameters, and model outcomes, population dynamics) and nature (the extent of epistemic versus inherent variability in HGT processes and outcomes). While acknowledging the larger perspective on uncertainty in our assessment of the ARM genes, the *ad hoc* group has identified a number of reducible uncertainties and knowledge gaps in the assessment of the biological effects of ARM

genes.

5.1. Uncertainty and knowledge gaps in the effects caused by human exposure to *proteins* encoded by ARM genes

The limited number of experimental studies available to resolve questions regarding the biological effects of human exposure to ARM gene encoded proteins results in an assessment that is based mainly on comparative experience and inference, rather than on direct experimental or epidemiological verification of the absence of effects. Some knowledge gaps identified include:

- With the possible exception of the NPTII protein, very little experimental evidence is available on the acute toxicity, allergenicity, or environmental stability of any protein conferring antibiotic resistance.
- Little experimental evidence is available to clarify the degradation kinetics or enzymatic activity of food-ingested proteins conferring antibiotic resistance.
- It is unclear how adequate current bioinformatics-based methodology is at assessing and predicting the allergenic potential of ARM gene products.
- It is unclear how adequately experimental investigations using purified ARM gene encoded proteins reflect the biological processes encountered by ARM gene encoded proteins present in foods with complex compositions.

These knowledge gaps can be categorized to the level and location of the uncertainty. The level of uncertainty reflects divergence in the conceptual understanding of the range of relevant biological effects caused by ARM gene encoded proteins exposed to mammalian cells. The location of uncertainty relates to divergence in the understanding of, and application of, models, methods and parameters to assess the pathways of the proteins in the GIT of mammals.

The *ad hoc* group is of the opinion that further developments in methodology, and standardization of methodology, should be encouraged to reduce uncertainty and strengthen the biological risk assessment of potential unintended effects caused by novel proteins produced in GMOs.

5.2 Uncertainty and knowledge gaps in the effects caused by human exposure to, or uptake of, intact ARM *genes*

Only a limited number of experimental studies are available to resolve questions related to direct human exposure to ARM genes. Some knowledge gaps identified include:

- There is a lack of a detailed understanding of uptake mechanisms, transport pathways and degradation mechanisms and dynamics of food-derived DNA in the intestinal cells and bloodstream of mammals.
- There is a lack of quantitative data on DNA exposure rates and fragment size distribution in the digestive system of various mammals digesting various foods.
- The extent to which ARM genes are taken up and transcribed in mammalian cell

cytoplasms is unclear.
- The proportion of food-derived ARM genes taken up into the bloodstream of mammals, and to what extent these will be transcribed if further transferred into host cells, is unclear.
- The effects of the enzymatic activity of ARM proteins in the cells of the GMO and in exposed mammalian cells needs to be further clarified.
- There is a lack of sensitive methodology and suitable model systems to address adequately the fate, and possible biological effects, of feed-derived genes, including ARM genes entering the bloodstream of mammals.
- There is a lack of demographic data on the relative exposure rates of mammalian cells to AR genes from bacteria naturally present in the GIT or in food, as compared to the exposure to ARM genes present in the genome of GMOs.

These knowledge gaps can be categorized to the level and location of the uncertainty. The level of uncertainty relates to divergence in the paradigms explaining the biological pathways and effects caused of ARM genes exposed to various mammalian cells. The location of uncertainty relates to the divergence in the understanding and application of models, methods and parameters used to assess the pathways of the ARM genes in the GIT and bloodstream of mammals.

The *ad hoc* group is of the opinion that further developments in the basic understanding of the pathways, distribution and degradation dynamics of all feed-derived DNA is necessary in order to strengthen the biological risk assessment of potential unintended effects caused by novel genes produced in GMOs. To obtain this information, further development of sensitive methodology is necessary.

5.3 Uncertainty and knowledge gaps in the effects of horizontal transfer of ARM genes to bacteria

Some experimental studies are available to resolve questions related to the indirect effects of ARM genes on human health after HGT to bacteria. Results from these experiments, together with comparative experience and inference, form the basis for our assessment of an HGT scenario. Experimental laboratory and field investigations suggest horizontal transfers of ARM genes from plants to bacteria are rare events. However, the experimental design of these studies has been questioned and, overall, our understanding of AR development in bacteria is currently more descriptive than predictive. The number of peer-reviewed studies on the *in situ* detection of natural transformation is limited, possibly indicating that the process occurs at very low frequencies under natural conditions. Alternatively, it has been suggested that valid conclusions cannot be drawn about the *in situ* activity of the process due to the lack of both adequate experimental design and sufficiently sensitive methodology (Heinemann et al., 2004, Nielsen and Townsend, 2004). Various knowledge gaps have been identified that relate to the potential indirect negative effects of unintended ARM gene transfer to bacteria:

- The proportion of intact ARM genes that are released from the cytoplasm of GMOs in various environments needs to be experimentally determined and a quantitative understanding of the degradation kinetics developed.

- The distribution, and tempo-spatial development, of competence for natural transformation must be determined in bacteria present in environments where ARM gene exposure occurs, and the factors governing the uptake of foreign DNA into bacteria should be further clarified. Studies of HGT processes in the GIT are of particular relevance.
- The concentration (copy numbers) of ARM gene homologues in natural environments, including the GIT of various mammals needs to be determined in order to generate a comparative understanding of the ecological importance of ARM gene donors.
- Antibiotic usage patterns need to be The factors determining the population dynamics of bacterial populations need to be identified and quantified in order to determine the possible biological significance, and relevant frequencies, of generating bacterial transformants carrying ARM genes.
- Further development of relevant biological model systems is necessary to resolve experimentally the possible occurrence of HGT of ARM genes into bacteria under representative environmental conditions.
- made publicly available to aid the identification of potentially selective environments for bacteria carrying ARM genes.

These knowledge gaps can be categorized to the level and location of the uncertainty. The level of uncertainty relates to divergence in the understanding of the ecological significance of the release, bacterial exposure, and uptake of ARM genes. The location of uncertainty relates to the divergence in the understanding and application of relevant models, methods and parameters used to assess the pathways of the ARM genes in various environmental sites. Moreover, there is a lack of quantitative data, and this generates uncertainty related to the occurrence of ARM gene homologues in the environment and the usage levels of relevant antibiotics in Europe.

The *ad hoc* group is of the opinion that further developments in the basic understanding of the molecular and population scale aspects of HGT processes in bacteria are necessary to strengthen the risk assessment of potential unintended effects caused by ARM genes in GMOs. Furthermore, experimental and epidemiological data are needed on the distribution of ARM gene homologues, resistance development, and antibiotic usage patterns in Europe.

6. Conclusions

The ARM genes used in GMOs are isolated from naturally occurring bacteria, thus a natural reservoir of these genes exists and any new introductions of AR genes through the release and use of GMOs must be seen in relation to the existing levels and locations in which AR genes reside naturally.

The limited number of experimental studies available to resolve the biological uncertainties arising from ARM gene introductions results in an assessment that is based mainly on comparative experience and inference, rather than on direct experimental or epidemiological verification of the absence of negative effects.
Most of the assumptions of a low risk produced by the release and exposure to ARM gene encoded *proteins* are based on inference from lack of observable effects from human and environmental exposure to naturally occurring counterparts to the ARM gene encoded proteins. Although the natural concentration of these resistance enzymes is often unknown, unintended consequences from natural exposure to these enzymes have not been reported. Thus, the *ad hoc* group has not identified any particular risk of Group 1 ARM gene encoded proteins in Norway. Due to the highly limited release of Group 2 ARM gene encoded proteins expected in Norway, the *ad hoc* group does not consider that the limited release of these proteins constitutes a health or environmental risk.

There are a number of knowledge gaps related to the uptake mechanisms, transport pathways and degradation dynamics of food-derived DNA in the bloodstream of mammals, and a lack of quantitative data on the degradation of DNA in different mammals digesting various sources of food. While acknowledging the lack of knowledge on these processes, the *ad hoc* group is of the opinion that mammalian exposure to ARM genes should be seen in relation to the daily exposure to a huge variety of genes (DNA) that mammals normally experience from any ingested food. The *ad hoc* group is not aware of any experimental evidence that suggests that ARM genes will produce negative effects if unintentionally taken up into, or produced in, mammalian cell cytoplasm. However, the possible interactions of ARM gene encoded proteins and their enzymatic activity with other proteins (proteome) in mammalian cytoplasms remains unexplored and should be further clarified. The *ad hoc* group has not identified any particular risks of the considered ARM genes, when examining the mammalian cell or cytoplasm exposure to feed-derived DNA containing ARM genes.

Some experimental studies are available to resolve questions related to the indirect effects of ARM genes on human health after HGT to bacteria. These experiments, together with comparative experience and inference from clinical resistance development, form the basis for our assessment of this scenario. Experimental laboratory and field investigations suggest HGT of ARM genes from plants to bacteria is rare. However, the experimental design of these studies have been questioned and, overall, our understanding of antibiotic resistance development in bacteria is currently more retrospective and descriptive, than predictive. Various knowledge gaps have been identified that relate to the process and outcomes of unintended ARM gene transfer to bacteria. Our current understanding of the release, stability and bacterial uptake of free DNA does not exclude the possibility that ARM genes can be exposed to competent bacteria, and may be acquired, at very low frequencies, by bacteria present in fields, food, and the digestive system

of mammals. Frequency estimates are, however, of limited utility to predict the long-term effect of sporadic gene transfer events. Knowledge of the selective conditions for rare bacterial transformants is necessary in order to predict the ecological and clinical outcomes of rare HGT events. Our current understanding of resistance development in pathogenic bacteria does not allow us to predict accurately the effect of introducing new sources of AR genes at various concentrations and locations.

As outlined in the EFSA opinion (2004), two criteria can be adopted to approximate and supplement the lack of a direct predictive capability. These are, 1) identification of the current natural reservoirs of resistance genes (section 3.3.3); and 2) identification of selective conditions favouring bacteria acquiring ARM genes (section 3.3.4). These assist in assessing the possible relative contribution of the ARM gene release from GMOs to the overall AR gene reservoir, and determining whether rare bacterial transformants that have acquired ARM genes are likely to undergo directional selection and amplification leading to clinically significant populations. Without a clear understanding of the ARM gene copy number released in various GMOs, and the corresponding copy numbers of naturally-occurring AR genes, it is difficult to estimate the relative contribution of ARM genes in GMOs to the overall AR gene exposure to bacteria.

Some data are available on phenotypic and genotypic resistance distributions among selected environmental samples and clinical isolates in various locations across Europe. However, no systematic studies are available that estimate the copy numbers of the various ARM gene homologues in various environments.

Group 1. Our literature survey identifies only few studies on the environmental distribution of the *npt*II gene. The limited data available suggests that the Group 1 gene *npt*II is only present in some natural and clinical bacterial isolates in Norway. The gene has been found in manure and sewage in a limited number of samples from the Netherlands and Germany. However, since the past and present agricultural usage of antibiotics varies considerably between countries, these observations need to be extended to Norwegian environments. The current usage pattern of aminoglycoside antibiotics in Norway and the low level of phenotypic resistance to aminoglycosides in pathogenic bacteria seen in Norway suggest caution should be applied in introducing new sources of resistance genes.
Little information is available on the distribution and ecology of the *hpt* gene, however, due to the limited clinical and veterinary importance of this gene, the *ad hoc* group has not identified specific concerns on the usage of this gene as an ARM. The *ad hoc* group is of the opinion that an increase in the prevalence of the *hpt*-gene will constitute a minimal risk, in agreement with EFSA's opinion.

The *ad hoc* group is of the opinion that an increase in the environmental exposure of the *npt*II-gene might constitute a somewhat larger risk than what is the case of the *hpt*-gene. The risk is nevertheless regarded as low. The reasoning for this view is that the *ad hoc* group considers that available data suggests the occurrence of this gene in pathogenic bacterial species in Norway to be low. Data on the overall copy number of the *npt*II gene would provide a comparative basis for the assessment of the potential risk related to the introduction of the *npt*II gene as an ARM gene in Norwegian environments. However, the lack of scientific studies does not allow the overall

copy number to be precisely estimated for most Norwegian environments. Changes in the pattern of antibiotic use, i.e. increased use of neomycin and/or kanamycin, as well as new information on *npt*II gene prevalence (i.e. relative copy number considerations) in relevant Norwegian environments may alter the risk assessment. Veterinary usage of aminoglycosides such as neomycin in Europe, including Norway, may create selective conditions for bacterial transformants harbouring ARM genes. The *ad hoc* group recommends that more quantitative data on the occurrence of the *npt*II gene (i.e. relative copy number considerations) in relevant Norwegian environments be collected to substantiate the assumptions made on the distribution of the gene.

Group 2. The $bla_{TEM\text{-}1}$ allele is unlikely to be present at high concentrations in natural environments in Norway. However, clinical studies of *E. coli* and other enterobacterial isolates suggest that the $bla_{TEM\text{-}1}$ gene is present in considerable quantities in the intestinal system of humans. Despite the apparent high prevalence of this gene among bacteria that are widely distributed in anthropogenic environments, the antibiotics to which the $bla_{TEM\text{-}1}$ gene confers resistance remain some of the most used in the world. The *str* gene has been identified across a range of habitats in Europe, although concentration estimates are rarely provided. Little information is available on the *cat* gene distribution among species and environments in Europe. The antibiotics to which the $bla_{TEM\text{-}1}$ gene and the *str* gene confer resistance are widely used in clinical and veterinary treatment of infections in Europe, including Norway (NORM/NORM-VET, 2004). The broad usage and utility of these antibiotics, combined with the observation of emerging ampicillin resistance in previously susceptible species, suggest caution should be applied in the dissemination of resistance genes in environments that are selective for bacterial transformants carrying ARM genes. The ad hoc panel is of the opinion that the expected low usage levels of the Group 2 ARM genes will not create an observable change in the clinical prevalence or importance of these genes in Norway.

Group 3. The *ad hoc* group adheres to assessment made in the EFSA opinion concluding that the ARM genes *npt*III and *tetA* should not be used in field trials or placed on the market.

7. References

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Appendix

The tables provide some examples of phenotypic resistance data collected by the NORM/NORM-VET surveillance programme in Norway (NORM/NORM-VET, 2004) See NORM/NORM-VET (2004) for definitions of resistance.

Abbreviations: S=sensitive, I=intermediate, R=resistant.

Table a. Antimicrobial resistance of *Escherichia coli* from human clinical isolates (blood cultures) in Norway 2000-2003.

Substance	Resistance (%)			
	2000	2001	2002	2003
Chlor-amphenicol	-	-	-	-
Ampicillin	28.0 (n=168)	28.1 (n=697)	26.5 (n=973)	30.1 (n=966)
Streptomycin	0.0	-	-	-
Gentamicin	1.2 (n=168)	0.7 (n=697)	1.2 (n=973)	0.6 (n=966)
Neomycin	0.0	-	-	-

Table b. Antimicrobial resistance of *Enterococcus* spp. from human clinical isolates (blood cultures) in Norway 2000-2003.

Substance	Resistance (%)			
	2000	2001	2002	2003
Chlor-amphenicol	-	-	-	-
Ampicillin	5.0 (n=121)	2.6 (n=191)	10.7 (n=252)	9.5 (n=252)
Streptomycin	14.9 (n=121)	19.3 (n=191)	23.1 (n=252)	15.5 (n=252)
Gentamicin	7.4 (n=121)	4.7 (n=191)	8.0 (n=252)	11.1 (n=252)
Neomycin	-	-	-	-

Table c. Antimicrobial resistance of *Enterococcus faecalis* from human clinical isolates (blood cultures) in Norway 2000-2003.

Substance	Resistance (%)			
	2000	2001	2002	2003
Chlor-amphenicol	-	-	-	-
Ampicillin	-	0.0 (n=155)	1.1 (n=188)	0.0 (n=183)
Streptomycin	-	12.3 (n=155)	19.1 (n=188)	14.2 (n=183)
Gentamicin	-	4.5 (n=155)	9.6 (n=188)	14.2 (n=183)
Neomycin	-	-	-	-

Table d. Antimicrobial resistance of *Enterococcus faecium* from human clinical isolates (blood cultures) in Norway 2000-2003.

Substance	Resistance (%)			
	2000	2001	2002	2003
Chlor-amphenicol	-	-	-	-
Ampicillin	-	16.7 (n=30)	57.4 (n=47)	50.0 (n=40)
Streptomycin	-	60.0 (n=30)	45.7 (n=47)	22.5 (n=40)
Gentamicin	-	3.3 (n=30)	4.3 (n=47)	2.5 (n=40)
Neomycin	-	-	-	-

Table e. Antimicrobial resistance of *Enterococcus faecalis* from animal and food in Norway 2000-2003

Substance	Sample	Resistance (%)			
		2000	2001	2002	2003
Chlor-	Bovine (meat)	-	0.0 (n=108)	-	0.0 (n=90)
amphenicol	Ovine (meat)	-	0.0 (n=78)	-	-
	Bovine (faecal)	-	0.0 (n=9)	-	-
	Poultry (meat)	0.0 (n=59)	-	0.0 (n=33)	-
	Poultry (faecal)	-	-	0.0 (n=133)	-
	Pork (meat)	1.9 (n=107)	-	0.0 (n=64)	-
	Porcine (faecal)	-	-	2.0 (n=59)	-
Ampicillin	Bovine (meat)	-	0.0 (n=108)	-	0.0 (n=90)
	Ovine (meat)	-	0.0 (n=78)	-	-
	Bovine (faecal)	-	0.0 (n=9)	-	-
	Poultry (meat)	0.0 (n=59)	-	0.0 (n=33)	-
	Poultry (faecal)	-	-	0.0 (n=133)	-
	Pork (meat)	0.0 (n=107)	-	0.0 (n=64)	-
	Porcine (faecal)	-	-	0.0 (n=59)	-
Streptomycin	Bovine (meat)	-	8.3 (n=108)	-	6.0 (n=90)
	Ovine (meat)	-	1.3 (n=78)	-	-
	Bovine (faecal)	-	0.0 (n=9)	-	-
	Poultry (faecal)	-	-	3.0 (n=33)	-
	Poultry (meat)	3.4 (n=59)	-	3.0 (n=133)	-
	Pork (meat)	16.8 (n=107)	-	3.0 (n=64)	-
	Porcine (faecal)	-	-	17 (n=59)	-
Gentamicin	Bovine (meat)	-	0.0 (n=108)		0.0 (n=90)
	Ovine (meat)	-	0.0 (n=78)		-
	Bovine (faecal)	-	0.0 (n=9)		-
	Poultry (meat)	0.0 (n=59)	-	0.0 (n=33)	-
	Poultry (faecal)	-	-	0.0 (n=133)	-
	Pork (meat)	0.9 (n=107)	-	0.0 (n=64)	-
	Porcine (faecal)	-	-	0.0 (n=59)	-
Neomycin	Bovine (meat)	-	0.0 (n=108)	-	1.0 (n=90)
	Ovine (meat)	-	0.0 (n=78)	-	-
	Bovine (faecal)	-	0.0 (n=9)	-	-
	Poultry (meat)	-	-	0.0 (n=33)	-
	Poultry (faecal)	-	-	3.0 (n=133)	-
	Pork (meat)	-	-	2.0 (n=64)	-
	Porcine (faecal)	-	-	2.0 (n=59)	-

Table e. continued. Antimicrobial resistance of *Enterococcus faecium* from animal and food in Norway 2000-2003.

Substance	Sample	Resistance (%)			
		2000	2001	2002	2003
Chlor	Bovine (meat)	-	0.0 (n=14)	-	0.0 (n=18)
amphenicol	Ovine (meat)	-	0.0 (n=9)	-	-
	Bovine (faecal)	-	0.0 (n=12)	-	0.0 (n=5)
	Poultry (meat)	0.0 (145)	-	0.0 (n=42)	-
	Poultry (faecal)	-	-	0.0 (n=116)	-
	Pork (meat)	0.0 (n=99)	-	0.0 (n=31)	-
	Pork (faecal)	-	-	0.0 (n=36)	-
Ampicillin	Bovine (meat)	-	0.0 (n=14)	-	0.0 (n=18)
	Ovine (meat)	-	0.0 (n=9)	-	-
	Bovine (faecal)	-	0.0 (n=12)	-	0.0 (n=5)
	Poultry (meat)	0.0 (145)	-	0.0 (n=42)	-
	Poultry (faecal)	-	-	0.0 (n=116)	-
	Pork (meat)	0.0 (n=99)	-	0.0 (n=31)	-
	Pork (faecal)	-	-	0.0 (n=36	-
Streptomycin	Bovine (meat)	-	0.0 (n=14)	-	0.0 (n=18)
	Ovine (meat)	-	0.0 (n=9)	-	-
	Bovine (faecal)	-	2.0 (n=12)	-	0.0 (n=5)
	Poultry (meat)	0.7 (145)	-	0.0 (n=42)	-
	Poultry (faecal)	-	-	0.0 (n=116)	-
	Pork (meat)	0.0 (n=99)	-	6.0 (n=31)	-
	Pork (faecal)		-	0.0 (n=36	-
Gentamicin	Bovine (meat)	-	0.0 (n=14)	-	0.0 (n=18)
	Ovine (meat)	-	0.0 (n=9)	-	-
	Bovine (faecal)	-	0.0 (n=12)	-	0.0 (n=5)
	Poultry (meat)	0.0 (145)	-	0.0 (n=42)	-
	Poultry (faecal)	-	-	0.0 (n=116)	-
	Pork (meat)	0.0 (n=99)	-	0.0 (n=31)	-
	Pork (faecal)			0.0 (n=36)	-
Neomycin	Bovine (meat)	-	0.0 (n=14)	-	0.0 (n=18)
	Ovine (meat)	-	0.0 (n=9)	-	-
	Bovine (faecal)	-	2.0 (n=12)	-	0.0 (n=5)
	Poultry (meat)	-	-	0.0 (n=42)	-
	Poultry (faecal)	-	-	0.0 (n=116)	-
	Pork (meat)	-	-	0.0 (n=31)	-
	Pork (faecal)	-	-	0.0 (n=36)	-

Table f. Antimicrobial resistance of *Escherichia coli* from animals and food in Norway 2000-2003.

Substance	Sample	Resistance (%)			
		2000	2001	2002	2003
Chlor-	Dog food (meat by-products)	4.5 (n=70)	0.8 (n=120)	-	-
amphenicol	Poultry (meat)	0.0 (n=204)	-	0.0 (n=155)	-
	Poultry (faecal)	-	-	0.0 (n=141)	-
	Poultry (clinical isolates)	-	-	0.0 (n=52)	-
	Pork (meat)	3.8 (n=158)	-	0.0 (n=137)	-
	Porcine (faecal)	-	1.1 (n=93)	<1 (n=187)	-
	Porcine (clinical isolates)	-	-	0.0 (n=39)	-
	Bovine (faecal)	-	0.0 (n=173)	-	<1 (n=120)
	Bovine (meat)	-	1.0 (n=100)	-	1 (n=90)
	Ovine (meat)	-	0.0 (n=100)	-	0 (n=118)
	Reindeer (faecal)	-	-	-	0.0 (n=42)
Ampicillin	Dog food (meat by-products)	10.4 (n=70)	4.2 (n=120)	-	-
	Poultry (meat)	10.8 (n=204)	-	7 (n=155)	-
	Poultry (faecal)	-	-	14 (n=141)	-
	Poultry (clinical isolates)	-	-	12 (n=52)	-
	Pork (meat)	8.9 (n=158)	-	<1 (n=137)	-
	Porcine (faecal)	-	4.3 (n=93)	6 (n=187)	-
	Porcine (clinical isolates)	-	-	13 (n=39)	-
	Bovine (faecal)	-	5.8 (n=173)	-	3 (n=120)
	Bovine (meat)	-	3.0 (n=100)	-	8 (n=90)
	Ovine (meat)	-	2.0 (n=100)	-	<1 (n=118)
	Reindeer (faecal)	-	-	-	0.0 (n=42)
Streptomycin	Dog food (meat byproducts)	20.9 (n=70)	7.5 (n=120)	-	-
	Poultry (meat)	12.3 (n=204)	-	2 (n=155)	-
	Poultry (faecal)	-	-	5 (n=141)	-
	Poultry (clinical isolates)	-	-	10 (n=52)	-
	Pork (meat)	23.4 (n=158)	-	21 (n=137)	-
	Porcine (faecal)	-	18.3 (n=93)	20 (n=187)	-
	Porcine (clinical isolates)	-	-	54 (n=39)	-
	Bovine (faecal)	-	13.3 (n=173)	-	13 (n=120)
	Bovine (meat)	-	7.0 (n=100)	-	17 (n=90)
	Ovine (meat)	-	2.0 (n=100)	-	2 (n=118)
	Reindeer (faecal)	-	-	-	24 (n=42)
Gentamicin	Dog food (meat by-products)	0.0 (n=70)	0.0 (n=120)	-	-
	Poultry (meat)	0.5 (n=204)	-	0 (n=155)	-
	Poultry (faecal)	-	-	<1 (n=141)	-
	Poultry (clinical isolates)	-	-	2 (n=52)	-
	Pork (meat)	0.6 (n=158)	-	0.0 (n=137)	-
	Porcine (faecal)	-	0.0 (n=93)	0.0 (n=187)	-
	Porcine (clinical isolates)	-	-	3 (n=39)	-
	Bovine (faecal)	-	0.0 (n=173)	-	0.0 (n=120)
	Bovine (meat)	-	0.0 (n=100)	-	0.0 (n=90)
	Ovine (meat)	-	0.0 (n=100)	-	0.0 (n=118)
	Reindeer (faecal)	-	-	-	0.0 (n=42)

Table f, continued. Antimicrobial resistance of *Escherichia coli* from animals and food in Norway 2000-2003.

Kanamycin	Dog food (meat by-products)	1.5 (n=70)	0.8 (n=120)	-	-
	Poultry (meat)	0.0 (n=204)	-	-	-
	Poultry (faecal)	-	-	-	-
	Pork (meat)	3.2 (n=158)	-	-	-
	Porcine (faecal)	-	-	-	-
	Bovine (faecal)	-	-	-	-
	Bovine (meat)	-	-	-	-
	Ovine (meat)	-	-	-	-
	Reindeer (faecal)	-	-	-	-
Neomycin	Dog food (meat by-products)	0.0 (n=70)	-	-	-
	Poultry (meat)	0.0 (n=204)	-	0.0 (n=155)	-
	Poultry (faecal)	-	-	<1 (n=141)	-
	Poultry (clinical isolates)	-	-	2 (n=52)	-
	Pork (meat)	1.0 (n=158)	-	0.0 (n=137)	-
	Porcine (faecal)	-	0.0 (n=93)	<1 (n=187)	-
	Porcine (clinical isolates)	-	-	0.0 (n=39)	-
	Bovine (faecal)	-	0.6 (n=173)	-	<1 (n=120)
	Bovine (meat)	-	0.0 (n=100)	-	0.0 (n=90)
	Ovine (meat)	-	0.0 (n=100)	-	(n=118)
	Reindeer (faecal)	-	-	-	0.0 (n=42)

-; not done