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Thematic Issue on Horizontal Gene Transfer

Review

Release and persistence of extracellular DNA in the environment

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The introduction of genetically modified organisms (GMOs) has called for an improved understanding of the fate of DNA in various environments, because extracellular DNA may also be important for transferring genetic information between individuals and species. Accumulating nucleotide sequence data suggest that acquisition of foreign DNA by horizontal gene transfer (HGT) is of considerable importance in bacterial evolution. The uptake of extracellular DNA by natural transformation is one of several ways bacteria can acquire new genetic information given sufficient size, concentration and integrity of the DNA. We review studies on the release, breakdown and persistence of bacterial and plant DNA in soil, sediment and water, with a focus on the accessibility of the extracellular nucleic acids as substrate for competent bacteria. DNA fragments often persist over time in many environments, thereby facilitating their detection and characterization. Nevertheless, the long-term physical persistence of DNA fragments of limited size observed by PCR and Southern hybridization often contrasts with the short-term availability of extracellular DNA to competent bacteria studied in microcosms. The main factors leading to breakdown of extracellular DNA are presented. There is a need for improved methods for accurately determining the degradation routes and the persistence, integrity and potential for horizontal transfer of DNA released from various organisms throughout their lifecycles.

Keywords: DNA release, stability, persistence or degradation / biosafety / GMO / lateral or horizontal gene transfer / natural transformation

INTRODUCTION

Extracellular DNA molecules are released and present in most terrestrial and aquatic environments. An accurate understanding of the processes and factors responsible for the release, persistence, and degradation of extracellular DNA[1] is important for several reasons. DNA present in, or released from, dead organisms represents a significant nutrient source that is both chemically uniform and ubiquitous for heterotrophic organisms. Genomic analyses of DNA from natural environments present unprecedented opportunities for increasing our understanding of natural microbial biodiversity and processes (Daniel, 2005). Such culture-independent studies of biodiversity and processes usually include extracellular DNA, and nucleotide sequences derived from dead specimens are also an important substrate for forensic analysis, conservation biology and monitoring of food ingredients derived from genetically modified organisms (GMOs). The study of the DNA of GMOs offers the potential for trackable systems for establishing the fate of living organisms or their remains. The environmental release of GMOs has also raised concerns over the potential environmental impact of recombinant DNA. This is because sequence heterogeneity and accessibility of the horizontally transferable pool of DNA present in a given environment influences, and may be the main factor that determines the evolutionary potential and trajectories of indigenous bacterial populations. An accurate prediction of the persistence and

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[1] We define extracellular, naked, free, ambient, or environmental DNA as: those molecules present in, or released from, cells in which energy production has permanently ceased, viral DNA, and DNA secreted from metabolically active cells.

pathways of environmental DNA will assist the biological risk assessment of GMOs.

PROCESSES LEADING TO RELEASE OF DNA MOLECULES

DNA molecules are exposed to the environment upon release from decomposing cells, disrupted cells, or viral particles, or *via* excretion from living cells. Actively secreted DNA is naturally more pure than DNA from dead cells, which is immersed in solutions of inorganic salts, proteins, RNA, cell membrane residues, polysaccharides, and other constituents of the cell cytoplasm and membrane. Release of intact DNA from decomposing cells depends on the activity and location of intracellular nucleases and reactive chemicals. In multicellular organisms, nucleases actively degrade host DNA in dead cells to release phosphorus, nitrogen, and sugars that nourish neighboring living cells (Nagata, 2005). In unicellular organisms, it is more difficult to establish communal behavior between decaying and living cells. Cell death terminates the cell's ability to synthesize nucleases and other enzymes. Thus, nuclease activity in dead cells will rely on the conditions for production of such enzymes produced or secreted by the cell prior to death. The nucleases also have to survive immediate digestion by proteases. Degradation of DNA in dead cells is a complex process that is dependent on a specific organism's physiology and the conditions leading to cell death. In general, intracellular DNA degradation appears to be a slow process, and, in most cases, extracellular DNA can be recovered from decomposing cells. In the following sections, the processes leading to the release of DNA from bacterial and plant sources are considered. The fate of extracellular DNA in food and the gastrointestinal tract will be reviewed in a separate study.

Release of DNA from bacteria

Bacterial DNA can be released either through active secretion by living cells or passive release from dead cells. DNA can also enter the environment indirectly, for example, in feces containing incompletely digested bacteria or in transducing phages. For instance, it takes approximately 4 h for rumen protozoa to digest any given bacterium in a rumen gut (Coleman, 1980). At this fast rate of bacterial turnover it is unclear how efficiently protozoa digest the various types of macromolecules present in the bacteria they engulf.

Many genera of bacteria, including *Acinetobacter, Alcaligenes, Azotobacter, Bacillus, Flavobacterium, Micrococcus, Neisseria* and *Pseudomonas* are known to release DNA during active growth (Dillard and Seifert, 2001; Hamilton et al., 2005; Lorenz et al., 1991; see also reviews by Lorenz and Wackernagel, 1994; Paget and Simonet, 1994; Thomas and Nielsen, 2005; Yin and Stotzky, 1997). Moreover, Matsui et al. (2003) demonstrated that extracellular plasmid DNA was released by *Escherichia coli* during co-cultivation with algae. *Deinococcus radiodurans* also harbors an excretion system for the active release of damaged DNA, during the repair of its genome after exposure to radiation (Battista, 1997), and this DNA excretion system shares sequence similarity with antibiotic excretion systems of actinomycetes (White et al., 1999). Most bacteria examined to date release DNA during growth *in vitro* (Lorenz and Wackernagel, 1994), but the extent and purpose of active DNA secretion by environmental bacteria is debated. Recent studies suggest extracellular DNA is an important component of bacterial biofilms (Steinberger and Holden, 2005; Whitchurch et al., 2002).

Bacterial cell death often leads to autolysis and release of cytoplasmic contents, including DNA (Palmen and Hellingwerf, 1995; 1997). DNA from lysed cells remains largely accessible to bacteria (Nielsen et al., 2000). Despite various laboratory studies, the actual amount of DNA released from autolytic bacterial cells is still unclear and largely depends on the conditions preceding cell death. For instance, Muela et al. (1999) reported a decrease in the chromosomal DNA content of *E. coli* during starvation in river water, indicating that ghost forms of bacteria with reduced chromosomal DNA content are generated during bacterial starvation. Similarly, Zweifel and Hagstrøm (1995) reported that only a minor fraction (2–32%) of the total bacteria counts obtained in soil by fluorescence staining methods contained detectable nucleoids. In contrast, more abrupt cell death, such as lethal exposure to antibiotics, can result in significant DNA release (Friedlander, 1975).

Substantial amounts of bacterial DNA are likely to be released into wastewater from inactivated microbial cultures in fermentation plants (Doblhoff-Dier et al., 2000). However, little information is available in the scientific literature on the exact treatment of biotechnological process-waste, and whether treatment efficiently removes or inactivates DNA in the microbial biomass. In a recent study, Andersen et al. (2001) reported that DNA fragments were present in waste products from a microbial fermentation plant. The biomass examined had been heat-treated at 90 °C and adjusted to pH 11 with CaO prior to field application as a fertilizer. Nevertheless, 3–9 μg bacterial DNA per mL was obtained with an average fragment size of < 2500 basepairs (bp), as determined by gel-electrophoresis. Fragment sizes of up to 1725 bp could be amplified by PCR, indicating that DNA of a biologically relevant size remained intact in the process waste.

Release of nuclear DNA from plants

Several studies have examined the persistence of plant DNA in the environment. However, little information is available on the molecular and cellular processes of release of DNA from agriculturally grown plants or dispersed plant material such as leaves, fruit, pollen, seeds, and shed root cap cells. The release of plant DNA can take place after (i) autolysis and decomposition of wilting tissues, (ii) mechanical disruption, or (iii) enzymatic degradation of cell structures by plant pathogens.

(i) The potential release of plant DNA from wilting tissues undergoing decomposition is limited by the activity of intracellular plant nucleases (Richards, 1987). During plant senescence, chlorophyll, proteins, RNA, and DNA are degraded in leaves (Green, 1994; Thomas and Stoddart, 1980; Woodhouse, 1982). However, the biochemical conditions prevalent in decomposing plant tissues may limit nucleolytic activity, which is dependent on water potential, pH, temperature, and salt concentration. For instance, nuclease activity is low in dried plant residues. Moreover, plant proteases can degrade nucleases, and other plant compounds such as polysaccharides can inhibit nuclease activity. The kinetics of DNA degradation in wilting plant material also depends on the activity of extracellular nucleases released from saprophytic bacteria and fungi. Despite the potential for DNA degradation from these various sources (Guan et al., 2005), Chiter et al. (2000) reported the recovery of intact high molecular weight DNA (> 20 kb) from maize silage and ryegrass silage, indicating that DNA present in harvested plant material, such as ensiled crops, does not necessarily undergo rapid degradation. Douville et al. (2007) detected a corn transgene in river water kilometers away from the cornfield. The rate of DNA degradation in decomposing cells of field grown plants depends on the circumstances of cell death and prevailing environmental conditions, and some environmental dissemination of transgene fragments must be expected.

(ii) Mechanical disruption of plant cell walls and membranes resulting from herbivores or farming practices, for example, may lead to the release of DNA. However, the abundant nucleases in plant cells can rapidly degrade DNA in liquid solution after cellular disruption and cell death (Garces et al., 2001; Gavrieli et al., 1992; Havel and Durzan, 1996; Ryerson and Heath, 1996). However, the extent of DNA degradation after mechanical disruption of agriculturally grown plant tissues with different water content should be further clarified. For example, Chiter et al. (2000) reported that grinding and milling of wheat seeds have little effect on the size of DNA molecules recovered.

(iii) Pathogenic bacteria often cause enzymatic degradation of plant structures, thereby possibly leading to the release of DNA from infected cells. Infecting bacteria release several types of enzymes that degrade cell walls, including endoglucanases, polygalacturonases, and pectin methylesterases (Bertolla et al., 2000). Due to enhanced enzymatic activity in plant cells responding to bacterial infection, it is unclear to what extent DNA from infected plant tissue remains intact and available to microbes. For instance, the simultaneous induction of plant and bacterial nucleases during infection may rapidly inactivate any DNA present. There are, however, some indications that free DNA is not broken down immediately in wounded plant tissue. Bertolla et al. (1999) injected purified plasmid DNA into tomato plants (*Lycopersicum esculentum)* infected with *Ralstonia solanacearum* cells. The plasmids were taken up by the plant pathogen, suggesting that competent bacteria might also be exposed to DNA released from plant cells. The circular plasmids used in the study may, however, be less prone to DNA degradation by exonuclease activity in infected plant tissues than linear DNA molecules. Restricted access to a DNA substrate could limit the disruptive effects of extracellular nucleases of saprophytic and pathogenic bacteria and fungi. In a study by the same group, light and electron microscopy were used to ascertain that physical contact between plant cell DNA and *R. solanacearum* bacterial cells is indeed feasible during the infection process (Bertolla et al., 2000).

Release of organelle DNA from plants

The potential for release of mitochondrial and plastid DNA differs from that of chromosomal plant DNA because organellar contents receive greater protection against cytoplasmic nucleases by additional membranes. It is known that organellar DNA is preserved in mammalian apoptosis. However, the nuclear and organellar transfer rate of mitochondrial and plastid DNA in eukaryotes suggests that organellar membranes do not greatly limit the horizontal transfer of organellar DNA on an evolutionary (Bergthorsson et al., 2003) or more immediate (Huang et al., 2003; Ricchetti et al. 1999; Yu and Gabriel, 1999) time scale. A few published studies describe processes that limit or induce DNA release from plastids or mitochondria (Nielsen et al., 2001; Thorsness et al., 1993; Vincent et al., 1988). Ceccherini et al. (2003) reported over 98% degradation of a chloroplast-harbored gene, as measured by PCR amplification, over a 72 h period in ground tobacco leaf material. Another study by the same group provided evidence that *Acinetobacter* sp. strain BD413 (formerly *A. calcoaceticus*, but recently renamed *Acinetobacter baylyi* (Vaneechoutte et al., 2006)) can take up chloroplast DNA released during its colonization of plant tissues. This indicates that bacteria (endophytic, saprophytic, and pathogenic) can absorb fragments of organelle DNA, despite the protection

of organelle membranes, when colonizing plant tissues (Kay et al., 2002). Follow-up studies would provide valuable information concerning the bacterial accessibility to DNA in chloroplasts used to produce foreign proteins (Daniell, 1999; Kahn and Maliga, 1999).

STABILITY OF DNA IN THE ENVIRONMENT

The recovery of DNA from archaeological and paleontological remains exemplifies the macromolecules' potential for long-term survival in the environment (DeSalle et al., 1992; Hofreiter et al., 2001; Iudica et al., 2001). DNA from specimens thousands of years old have been successfully amplified by PCR (see Austin et al., 1997; Landweber, 1999 for an overview). Analysis of intact DNA extracted from small, degraded specimens and tissue samples has also become a valuable tool for criminal and conservation forensics (Landweber, 1999). Moreover, DNA extracted from processed food is used for detection and quantification of GM ingredients (Miraglia et al., 2004). It is evident that DNA fragments can resist some environmental conditions and persist in various tissues and locations over a long time. However, DNA that remains stable over extended time must be protected from bacterial degradation and is probably not directly available to competent bacteria.

Persistence of DNA in soil

Soil is an environment rich in nucleic acids that seem most concentrated in the upper soil layers (Baker, 1977). It has been estimated that up to 70% of the DNA molecules present in soil is of fungal origin (Borneman and Hartin, 2000; Smit et al., 1999; van Elsas et al., 2000). Typically, around 80 μg of DNA can be isolated per gram soil (Niemeyer and Gessler, 2002; Paget et al., 1998; Torsvik and Goksøyr, 1978). The extracellular bacterial fraction thereof is difficult to quantify because methods applied for DNA desorption from soil disrupt intact cells, leading to the release of DNA. However, it has been estimated that between 0.03 μg (Selenska and Klingmüller, 1992) to 1 μg (Ogram et al., 1987) of DNA per gram of soil is present in an extracellular form. Nucleases rapidly hydrolyse DNA added to soil in excess amounts (*e.g.* >10 μg per g soil) (Greaves and Wilson, 1969; Romanowski et al., 1992; 1993; Widmer et al., 1996). Blum et al. (1997) observed that the majority of nuclease activity in soil is of bacterial origin. Despite the presence of DNA degrading enzymes, fragments of DNA have been found to persist in agricultural soils for extended periods of time, as demonstrated by PCR amplification of targets up to approximately 1000 bp in length (Tab. 1).

Persistence of bacterial DNA in soil. The kinetics of DNA degradation in soil bacteria has been studied in laboratory microcosms using live inocula, suspensions of dead bacterial cells, or purified DNA. Whereas many of these studies have focused on the physical persistence of DNA in soil, as measured by DNA isolation, hybridization, and PCR, some have addressed the ability of such DNA to transform bacteria using transformation assays and selective plating (Tab. 2).

DNA in live donor bacteria. Several studies have determined the fate of plasmid or chromosomal DNA present in live bacterial inocula that have been introduced into soil but compete poorly with indigenous bacterial populations (see Tab. 2). Henschke et al. (1991) inoculated an *E. coli* strain harboring a pUC19 derivative into soil to determine the persistence of the plasmids. The inoculum of $\sim 10^8$ CFU per gram soil could be detected for up to 25 days. A corresponding loss of signal was found in total DNA isolated from the soil after amplification of an 800 bp fragment with plasmid-specific primers, indicating that the presence of the plasmid corresponded closely to host cell viability. Similarly, Selenska and Klingmüller (1991) introduced *Enterobacter agglomerans* into a loamy sand soil (10^7 CFU per gram soil) and obtained signal from the inoculum after 70 days by radioactive hybridization of extracted total DNA, while viable cells were only detected for up to 63 days. A later study by Recorbet et al. (1993) provided evidence that, in some cases, chromosomal DNA persists longer in soil than detectable host cell viability suggests. The study investigated the persistence of DNA in soil microcosms inoculated with an *E. coli* strain. The population declined below the detection limit after 15 days. However, most-probable number PCR yielded positive signals after 40 days. England et al. (1997) investigated the persistence of chromosomal DNA from *Pseudomonas aureofaciens* in soil microcosms by PCR amplification of a 630 bp fragment. Positive signals were obtained up to 4 weeks after inoculation of a heat-killed cell suspension suggesting that extracellular DNA can persist in soil for extended periods of time.

The above studies indicate that, as more sensitive molecular techniques are applied, DNA, presumably in an extracellular form, can be detected for weeks in inoculated soil microcosms. However, the exact location of the DNA remains elusive. For instance, if the inoculum develops a viable but non-culturable state in response to soil conditions, the number of live cells, estimated from the number of colony forming units (CFUs) recoverable from soil, may soon drop below the detection limit, while their DNA is still detectable by PCR or radioactive hybridization.

Using *in situ* transformation assays to determine the accessibility of chromosomal DNA in live bacterial

Table 1. Some studies of the persistence of plant DNA in soil.

Source and initial status of DNA	Soil type	Genes monitored	Detection method	Period detected	Reference
Ground tobacco leaf tissues added to soil microcosms	Silt loam	Nopaline synthase and 35S CaMV promoters	Extraction of total DNA, PCR	120 days	Widmer et al., 1996
Tobacco leaves added to soil, potato litter on soil surface	Silt loam	Nopaline synthase and 35S CaMV promoters	Extraction of total DNA, PCR	77–137 days	Widmer et al., 1997
Field sites with transgenic tobacco plants	Sandy clay-loam	*aac*I – gentamycin resistance	Selective plating, extraction of total DNA, PCR, hybridization	1 year	Paget et al., 1998
Soil microcosms with added transgenic sugar beet DNA (purified)	Silt loam	*npt*II – kanamycin resistance	Extraction of total DNA, PCR	3–6 months	Gebhard and Smalla, 1999
Field sites with transgenic sugar beet plants	Silt loam	*npt*II – kanamycin resistance	Selective plating, extraction of total DNA, PCR, hybridization	2 years	Gebhard and Smalla, 1999
Poplar tree leaves	Forest soil	*npt*II – kanamycin resistance	Extraction of total DNA, PCR	4 months	Hay et al., 2002

Table 2. Some studies of the persistence and transforming activity of bacterial DNA in soil microcosms.

Source and initial status of DNA in soil microcosm	DNA monitored (location)	Soil used (nonsterile)	Detection method	Stability	Reference
Live inoculum of *Escherichia coli*	pUC19 derivative	Parabrown earth	Hybridization, PCR	28 days[a]	Henschke et al., 1991
Live inoculum of *Enterobacter agglomerans*	TN5 transposon (chromosomal and plasmid)	Sandy loam	Hybridization	70 days[a]	Selenska and Klingmüller, 1991; 1992
^{3}H-labeled plasmid pUC8-ISP	pUC8-ISP *amp*^{R}	Loamy sand, clay, silty clay	Radioactive quantification, slot and Southern blot, transformation assay	> 10 days	Romanowski et al., 1992
Live inoculum of *Escherichia coli*	*Uid*A and *npt*II genes (chromosomal)	Silt loam	MPN PCR	40 days[a]	Recorbet et al., 1993
Plasmid pUC8-ISP	pUC8-ISP *amp*^{R}	Loamy sand, clay, silty clay	PCR and electroporation of *E. coli* cells	60 days	Romanowski et al., 1993
Purified DNA from *Bacillus subtilis* adsorbed to clay	pHV14 and amino acid markers (chromosomal)	Sandy loam	Transformation assay	15 days	Gallori et al., 1994
Cell lysates of *Pseudomonas aurefaciens*	Tn7-lac fusion element (chromosomal)	Sandy loam	PCR	30 days	England et al., 1997
Purified DNA from *Acinetobacter* sp.	*npt*II gene (chromosomal)	Silt loam, loamy sand	Transformation assay	30 min	Nielsen et al., 1997a
Purified DNA from *Pseudomonas stutzeri*	pSI1 (with inserted chromosomal homology)	Loamy sand	Transformation assay	3 days	Sikorski et al., 1998
Cell lysates of *Acinetobacter* sp., *Pseudomonas fluorescens*, *Burkholderia cepacia*	*npt*II gene (chromosomal)	Silt loam	Transformation assay	4–8 h	Nielsen et al., 2000

[a] Equal to (Henschke et al., 1991) or longer than (Recorbet et al., 1993; Selenska and Klingmüller, 1991) the period the introduced bacterial inoculum could be detected.

inocula, Graham and Istock (1978; 1979) were the first to report the uptake of naked DNA by natural transformation in sterile potting soil. Cells of the soil bacterium *Bacillus subtilis* were used as recipients of chromosomal DNA initially present in a co-inoculated *B. subtilis* donor strain. Similar studies using *B. subtilis* as a recipient strain of chromosomal DNA in soil have also been reported by Lee and Stotzky (1990; 1999). Whereas studies of natural transformation with DNA released from live donor cells provide evidence that DNA initially present in bacterial cells subsequently become available to competent bacteria, the precise horizontal transfer pathways taken by the DNA from the donor to the recipient bacterium remain unresolved. Sterile soil systems readily permit the identification of transformants in soil without introducing background problems associated with indigenous microflora. However, in nonsterile systems, DNA degradation can occur more rapidly, thereby shortening the time period during which gene transfer can occur (Nielsen et al., 1997a; 1997b; 2000).

DNA in bacterial cell lysates. Using heat-inactivated cell lysates rather than live cells, Nielsen et al. (2000) determined the time period that chromosomal DNA present in lysates of *Acinetobacter* sp., *Pseudomonas fluorescens,* and *Burkholderia cepacia* cells remained accessible to bacteria in sterile and nonsterile silt loam soils. The cell lysates were found to persist and remain accessible for uptake by competent *Acinetobacter* sp. cells up to 4 days after incubation in sterile soil. In nonsterile soil, transforming activity was limited to 4–8 h. Because comparable amounts of purified DNA retained transforming activity for a shorter period in the same soil system, it appears that cell lysates generally do not inhibit transformation, and that DNA may be protected from rapid degradation within the lysed cell suspension (Nielsen et al., 1997a; 1997b).

Purified bacterial DNA. Several research groups have determined the time period during which purified chromosomal DNA remains accessible to competent bacteria in soil (Tab. 2). Two studies by Romanowski et al. (1992; 1993) examined the persistence of plasmid DNA introduced into three different nonsterile soil types using several methods. The DNA (2 μg per 10 g of soil) was detected for up to 60 days at up to 0.2% of the initial concentration depending on soil type. The artificial transformation and electroporation assays used indicated that the plasmid DNA did not suffer irreversible damage from soil incubation. Gallori et al. (1994) used natural transformation assays to investigate the stability of clay-adsorbed DNA in soil samples. They found that clay-bound plasmid and chromosomal DNA of *B. subtilis* transformed competent *B. subtilis* cells after 15 days incubation in soil. In a later study, Nielsen et al. (1997a) investigated the availability of purified chromosomal DNA to competent *Acinetobacter baylyi* strain BD413 cells in soil microcosms. DNA added to loamy sand was less accessible to competent bacteria than DNA added to a silt loam soil. Sikorski et al. (1998) added plasmid DNA in a nonsterile loamy sand soil microcosm and could detect transforming activity of the soil-incubated plasmid DNA on *Pseudomonas stutzeri* for up to 3 days.

The microcosm studies listed in Table 2 suggest that minor fractions of bacterial DNA released from living or dead cells can persist in soil over biologically significant periods and competent bacteria can access such DNA. There is a sharp contrast between the time period (up to 70 days) that DNA present in dead cells, in lysates, or as purified molecules can be detected in physical assays such as Southern blotting and PCR, and the few hours (chromosomal) or days (plasmids) that introduced DNA is available to bacteria at detectable levels during *in situ* transformation assays.

Persistence of plant DNA in soil. Organic material from plants, including DNA, is continually introduced into soil. Lynch (1983) estimated that the relative amounts of carbon introduced into agricultural soil per hectare per year derives from root decomposition, 400 kg; root exudation, 240 kg; straw residues, 2800 kg; and autotrophic microbes 100 kg. Below the plough layer, root exudation and plant residue decomposition result in further release of organic material (Lynch, 1983). Plants are major contributors of organic material, including DNA (Arumuganathan and Earle, 1991), into soil. The contributions of plant and microbes to soil organic matter have been reviewed by Kögel-Knabner (2002). Several studies have been conducted on the stability of plant DNA in soil (see Tab. 1). The majority of these studies have monitored the persistence and stability of transgenes in plant DNA, due to the ease of detection and selection of inserted marker genes. The degradation rate of transgenic plant DNA is expected to be identical to that of the conventional variety, unless the transgenes cause phenotypic changes that influence the decomposition process in the plant tissue. The stability of chromosomal plant DNA has been investigated both in soil microcosms and under natural conditions. All of the published studies have reported a gradual decrease in the quantity and size of DNA over time (Tab. 1).

Widmer et al. (1996) added transgenic ground tobacco leaf tissue to a silt loam soil microcosm and were able to detect its recombinant DNA marker for up to 120 days after addition to soil. Widmer et al. (1997) also performed the first field study of the persistence of transgenic plant DNA in soil. The stability of DNA in tobacco leaves buried to a 10 cm depth in soil and of DNA from potato stems, leaves, and tuber litter decomposing on the soil surface of a loamy sand, were studied using the same transgenic marker approach. DNA from composted

tobacco leaves was detectable for up to 77 days and for 137 days in the potato litter. The transgenic plant DNA could not be PCR amplified from the adjacent bulk soil, indicating that the majority of the plant DNA was contained within the plant residues and litter.

In a later study, Paget et al. (1998) developed a transgenic tobacco line to determine the stability of plant DNA in the field and to monitor putative transfer of the plant transgene to indigenous soil bacteria. Tobacco plants that contained a chromosomally inserted gentamicin resistance gene were grown in a sandy clay-loam soil at field sites in France, and the persistence of the DNA was monitored with three different primer sets. The persistence of the plant DNA was monitored for 3 years and positive PCR signals were detected for up to 1 year. It was unclear if the plant DNA that gave rise to PCR signals for up to 1 year was preserved as extracellular fragments in soil or resided in decomposing plant residues.

Using a similar approach, Gebhard and Smalla (1999) monitored both the stability of transgenic sugar beet (*Beta vulgaris*) DNA, and the possible horizontal transfer of its DNA to indigenous bacteria in soil. Total DNA was extracted from a silt loam soil from field trials in Oberviehhausen, Germany and amplified with three different primer sets specific for transgenic sugar beet DNA. PCR amplification and subsequent Southern blot analysis yielded positive signals for up to 2 years after initial farming of the sugar beets. Gebhard and Smalla (1999) also investigated the stability of sugar beet DNA in soil microcosms by PCR amplification, blotting, and radioactive hybridization to a gene-specific probe, and reported that fragments of purified DNA could be detected after 6 months of incubation. Examining the persistence of DNA in radicles of sugar beet plants and chicory plants, Degand et al. (2002) were able to obtain positive PCR/hybridization signals for 25 or 50 days, respectively. In a first study to investigate the persistence of DNA in decomposing tree leaf material, Hay et al. (2002) could detect PCR amplifiable DNA fragments for up to 4 months. The longest detection period was found for leaves incubated in mesh-bags resting on weeds above the soil.

In summary, DNA molecules from agriculturally grown plants have been detected in soil for extended periods of time after harvest. Estimated turnover time of microbial biomass carbon in soil ranges from several months to over 2 years (Couteaux et al., 2002; Ladd et al., 1996; Lynch, 1983). The studies in Table 1, which report the stability of plant DNA in natural soils for up to 2 years, concur with these calculations. The form and location in which plant DNA generally persists in agricultural environments is currently unclear. Protection of plant DNA from rapid enzymatic degradation after binding to soil particles or within dried plant material where enzymatic activity is inhibited has been suggested (Widmer et al., 1997). The long-term physical persistence of plant DNA, as detected by PCR analyses, does not reflect the short-term activity of plant DNA in bacterial transformation assays in soil microcosms. As is the case for bacterial studies, purified plant DNA added to soil seems to experience a shorter lifespan than DNA within cellular material, possibly due to a lack of protection offered by intact plant cell walls and membranes.

Persistence of DNA in water and sediment

Cell-free DNA is an important component and nutrient source in water and sediment (Boehme et al., 1993; Dell'Anno and Corinaldesi 2004; Dell'Anno and Danovaro, 2005; Dell'Anno et al., 2002). Significant concentrations of dissolved DNA have been found in marine water, freshwater and sediments at concentrations from 1 μg to above 80 μg dissolved DNA per L (see Tab. 5 in Karl and Bailiff, 1989). Because it is difficult to distinguish among DNA present as pure molecules, encapsulated in viral particles or ultramicrobacteria (< 0.2 μm), and bound to colloids, the term dissolved DNA is frequently used to encompass these forms as well as free DNA molecules. Jiang and Paul (1995) characterized dissolved DNA in summer seawater samples from the Gulf of Mexico and estimated that approximately half of the dissolved DNA in the marine environment existed as free DNA molecules and the remaining half as molecules associated with viral particles and, potentially, colloids. Of the bound form, 17–30% was of viral origin and the remainder came from bacterial and eukaryotic sources. Thus, a significant fraction of dissolved DNA in aquatic environments is likely to exist in free form.

Persistence of DNA in marine water and sediment. Estimates of marine concentrations of dissolved DNA range from 0.03 to 88 μg per L, and decrease as a function of distance from shore and the depth of the water column (DeFlaun and Paul, 1989; DeFlaun et al., 1986; 1987; Karl and Bailiff, 1989; Paul and Myers, 1982; Paul et al., 1987). For instance, Pillai and Ganguly (1970; 1972) found 13 to 24 μg DNA per L in Bombay Harbor, India, and DeFlaun et al. (1987) found between 0.2 and 19 μg DNA per L in samples from the Gulf of Mexico. Jørgensen and Jacobsen (1996) reported concentrations of 2 to 11 μg dissolved DNA per L in estuarine mesocosms. In their study, the addition of ^{3}H labeled DNA to water samples revealed rapid incorporation rates of up to 0.9 μg DNA per L per h in bacterioplankton (Jørgensen and Jacobsen, 1996). Growing heterotrophic bacterioplankton appears to be a major source of dissolved DNA in subtropical estuarine and oligotrophic oceanic environments presumably releasing DNA after grazing, cell

death, and lysis (Paul et al., 1987). Experiments with labeled bacterial chromosomal DNA, however, show that it is rapidly hydrolyzed by both extracellular and cell-bound nucleases and taken up by indigenous microbial populations (Paul et al., 1987). DNA turnover times vary considerably; they are as short as 6.5 h for estuarine samples from Bayboro harbor, Tampa Bay, USA (Paul et al., 1987), whereas other studies estimate DNA turnover times of 10 days to 2 months (Bazelyan and Ayzatullin, 1979). DeFlaun and Paul (1989) examined the degradation of an *E. coli* plasmid in eutrophic estuarine water. Intact plasmids (added at 15 ng per mL water) were detectable after 4 h using agarose gel-electrophoresis and for up to 24 h by hybridization to a plasmid specific probe. Such gradual degradation rates may not greatly limit the uptake of DNA by competent bacteria in water (DeFlaun and Paul, 1989).

Dupray et al. (1997) determined the degradation rates of the virulence plasmid of *Salmonella typhimurium* in microcosms of autumnal and summer seawaters sampled from offshore Brittany, France. Both free DNA and dead *Salmonella* cells were introduced into 2 L microcosms at initial concentrations of 10^5–10^6 heat-killed cells per mL water or 14–22 ng purified DNA per mL water. DNA in the dead cell suspension was detected for up to 55 days in autumnal seawater maintained at 10 °C and 10 days in summer seawater. Shorter stability of DNA was observed at 20 °C. Free DNA was less stable and persisted from 3 to 8 days at 10 °C and 2 to 4 days at 20 °C, as measured by PCR. The DNA was detectable for up to 3 months in control microcosms containing 3 μm-filtered seawater suggesting that it is aquatic microorganisms that normally degrade DNA. Similarly, Palmer et al. (1993) found that bacterial DNA of *Legionella* sp. was degraded within 4 days in warm seawater (16 °C).

Fewer studies are available on the turnover rate of DNA in marine sediments. Ogram et al. (1987) estimated that around 3–4% of the DNA in sediment is extracellular, however, more recent studies suggest the majority of DNA in sediments are present in an extracellular form (Corinaldesi et al., 2005). Novitsky (1986) estimated a DNA turnover rate in marine sediment of 20 days based on studies using radioactively labeled bacteria. Thus, slower degradation kinetics likely prevail in marine sediments than in marine water.

Persistence of DNA in freshwater and sediment. The typical concentration of extracellular DNA in freshwater has been estimated to range from 1 to 17 μg DNA per L water, though wider ranges have been reported (Lorenz and Wackernagel, 1994; Siuda and Gude, 1996). Minear (1972) measured between 4 and 30 μg extracellular DNA per L in lake water. A study by Beebee (1993) revealed two fractions of extracellular DNA in natural freshwater: one fraction of > 20 kb in size was mainly of bacterial and viral origin, while the other fraction contained smaller molecules of 100 to 500 bp of unknown origin. Alvarez et al. (1996) examined the stability of plasmid DNA in samples of distilled, dechlorinated tap, marine, and river water from Puerto Rico. Whereas plasmid DNA (10 μg per mL) in distilled and tap water remained stable for 5 days at room temperature, the plasmids added to the marine and river water could only be detected for up to 24 h, as measured by hybridization with a radiolabeled probe. Kim et al. (1996) also reported a comparably short persistence time for recombinant plasmid DNA added to nonsterile creek water. Using samples of stratified lake water from Lake Biwa in Japan, Matsui et al. (2001) reported that added plasmid DNA was completely degraded within a week in lake surface water, however, DNA did not degrade so rapidly in samples collected from deeper waters. A more recent study by England et al. (2005) reported that high concentration of purified viral DNA added to an outdoor aquatic microcosm disappeared within 24 h. Deere et al. (1996) studied the stability of extracellular DNA released from living *Aeromonas salmonicida* cells in natural nonsterile microcosms consisting of lake water and lake sediment. The survival of cells and DNA was detected for 4 weeks in the water fraction, and stability was similar in the loamy sediment. However, in the sandy sediment, the inoculant could be detected for an additional period of 3 weeks. Despite the absence of viable cells, the DNA remained amplifiable by PCR for up to 13 weeks. The authors speculated that the decreased detection of DNA in open water was due to adsorption to sediment as well as to its degradation. Douville et al. (2006) detected a corn transgene in water and sediment microcosm for up to 21, and 40 days, respectively, and also reported transport of the transgene from the field corn site in river water to locations several kilometers away.

The observed long-term persistence of DNA fragments in water environments, despite the lack of recoverable initial host inoculum cells, mirrors results in soil environments, where DNA signals can be obtained by PCR for weeks after the inoculum loses viability. Most studies to date conclude that extracellular DNA molecules are present in both marine and freshwater environments, though degradation of introduced naked DNA usually occurs within only hours or days.

FACTORS AFFECTING THE STABILITY OF EXTRACELLULAR DNA

Many factors affect the chemical and physical integrity of DNA in various environments. Extracellular DNA may be degraded by nucleases, chemically modified, sheared, or stabilized by binding to mineral surfaces or humic substances. In the following sections, some key factors

influencing the persistence of DNA in the environment are discussed.

Enzymatic degradation of DNA

The enzymatic degradation of organic material by saprophytes causes the breakdown of the majority of extracellular DNA present in the environment (DeFlaun et al., 1987; Nygaard, 1983). The enzymes responsible for the degradation of DNA, DNases, are found in most microbial habitats (Blum et al., 1997; Novitsky, 1986; Paul et al., 1989; 1990; Turk et al., 1992). DNases ultimately convert DNA to deoxyribose, inorganic orthophosphate, and purine and pyrimidine bases, enabling microbes to use DNA as a source of carbon, nitrogen, phosphorus, and nucleic acid precursors. Though pure nucleic acids are generally not sufficient as a sole carbon source for bacteria, the soil bacterium *Serratia marcescens* and the intestinal bacterium *E. coli* are capable of utilizing DNA exclusively for carbon (Beliaeva et al., 1976; Benedik and Strych, 1998; Finkel and Kolter, 2001). Depending on their mode of action, nucleases that degrade DNA substrates are classified as sugar-specific deoxyribonucleases (exo-deoxyribonucleases, endo-deoxyribonucleases and restriction endonucleases), or sugar non-specific nucleases (endonucleases and exonucleases) (Rangarajan and Shankar, 2001). Both single- and double-stranded nucleases are widespread (Desai and Shankar, 2003). However, their expression levels and spatial distribution in natural environments often remain unclear.

Microbially encoded nuclease activity may be extracellular, associated with the cell surface (Puyet et al., 1990), or intracellular. Contrary to common belief, extracellular nucleases are rare, and have only been observed in association with a small number of bacterial species (Benedik and Strych, 1998). The *S. marcescens* extracellular nuclease has been comprehensively studied (Benedik and Strych, 1998; Eaves and Jeffries, 1963; Yonemura et al., 1983). *S. marcescens* and other *Serratia* species are readily isolated from soil, water, plants, and animals (Ahrenholtz et al., 1994b; Grimont and Grimont, 1991). Both the *S. marcescens* nuclease and DNase I isolated from the bovine pancreas have endonuclease activity and produce oligonucleotides of 3 to 5 bp in length (Cuatrecasas et al., 1969).

Nuclease activity in soil. Enzymatic activity in soil depends on cellular enzymes located in active or dormant cells, dead cells, or cell debris and on extracellular enzymes existing free in solution or adsorbed by soil colloids or humic materials (Burns, 1982; Ladd, 1978; Nannipieri, 1994; Nannipieri et al., 2002; Oades, 1988; Pietramellara et al., 1997). In particular, the activity of extracellular enzymes stabilized by soil colloids is important, because they often resist environmental stress factors that affect the activity of enzymes harbored by microorganisms (Nannipieri et al., 2002). Unadsorbed extracellular nucleases are rare and those present are believed to be short-lived in soil due to the presence of proteases. Purified DNA and DNA from cell lysates added to soil remain intact over a longer period of time in sterile soils than in nonsterile soils (Greaves and Wilson, 1969; Nielsen et al., 1997a; 1997b; 2000). Blum et al. (1997) suggested that nucleases associated with the active microbiota are responsible for most of the observed degradation in nonsterile soil. Interestingly, the addition of nucleases to soil causes little degradation of extracellular DNA, presumably due to the rapid inactivation of the nucleases after binding to soil substances (Demanèche et al., 2001; Graham and Istock, 1978; Harter and Stotzky, 1971).

Nuclease activity in water and sediment. A number of studies have addressed the presence and activity of nucleases in water and sediment (Bazelyan and Ayzatullin, 1979). Using a fluorometric method, Maeda and Taga (1973) detected DNase activity in natural seawater and sediment samples taken from Tokyo Bay, Japan. Later, they also reported that nucleic acid hydrolyzing bacteria are distributed in seawater and sediment in the Pacific Ocean at up to 100 CFU per mL (Maeda and Taga, 1974). The majority of the DNase activity was found on suspended particles and microbial cells rather than dissolved in seawater. Ruiz et al. (2000) reported the presence of high nuclease activity in anaerobic samples of freshwater and marine sediment from locations in Mexico, while Ahrenholtz et al. (1994a) show that nuclease acivity in groundwater is much higher at 37 °C than at 4 °C. Lorenz and colleagues (Lorenz and Wackernagel, 1987; Lorenz et al., 1981) reported that DNA bound to sea sand is more protected than DNA in the water column, suggesting that marine sediments offer extracellular DNA protection against immediate degradation through reduced nuclease accessibility. Thus, based on these reports, we can hypothesize that extracellular nucleases share similar fates in sediment and in soil, since both environments offer adsorbant surfaces such as clays, oxides, and hydroxides, and are capable of entrapping molecules such as proteins and nucleic acids in the humic matrix. In contrast, adsorption or entrapment of such molecules in water would occur less frequently because the environment is less structured and more homogeneous than soils and sediments.

Other factors affecting DNA integrity

Chemical or physical inactivation of DNA. Numerous physical conditions and chemical compounds severely compromise the integrity of DNA molecules exposed to the extracellular environments. Most importantly, DNA

repair enzymes that are active outside cells are unknown. Thus, no longer maintained by cellular DNA repair mechanisms, the DNA molecules will accumulate environmentally inflicted damage.

Exposure to high temperatures leads to single-stranded and fragmented DNA molecules (Bauer et al., 2003). Single-stranded DNA seems to be an inefficient substrate for natural transformation (Lorenz and Wackernagel, 1994). If conditions are appropriate for subsequent reassociation, functional double-stranded DNA molecules may be restored, but reassociation of complex mixtures of single-stranded DNA molecules may be an exceedingly slow process that requires several days (Torsvik et al., 1990). The presence of other contaminating macromolecules may further slow or block the process. On the other hand, single-stranded DNA may be protected from some nucleases, including restriction enzymes. Chiter et al. (2000) reported that exposure to a temperature of 95 °C for only 5 min was sufficient to heavily fragment DNA present in plant tissues. The same DNA remained intact for increasing amounts of time when lower temperatures, such as 90 °C, were applied for 30 min. Bauer et al. (2003) reported that fragments of 1339 bp could be detected by PCR after 10 min boiling of raw soymilk. Fragmented DNA also results from sterilization by autoclaving (Chiter et al., 2000). Rizzi et al. (2003) found that 40% of a corn DNA target could be detected by real-time-PCR assays in a water solution after 1 h treatment at 99.9 °C. However, agarose gel electrophoresis showed extensive fragmentation of the DNA. Nielsen et al. (2000) reported that autoclaved chromosomal DNA was unable to transform highly competent *Acinetobacter baylyi* cells. However, treatment at 80 °C for 15 min yielded dead cells and the DNA in the cell suspension were still active in transformation assays. Conversely, incubation at lower temperatures indirectly benefits the stability of DNA molecules by decreasing the nucleolytic activity of enzymes and other reactive chemicals as well as the growth of saprophytic bacteria. Nevertheless, development of competence and uptake of genes by natural transformation can occur at low temperatures that are sub-optimal for bacterial growth (Lorenz and Wackernagel, 1994; Nielsen et al., 1997a).

The integrity of free DNA molecules is vulnerable to the direct and indirect effects of radiation. Gamma-irradiation, used for sterilization of food or feedstuff, leads to fragmented and biologically inactivated DNA. Many chemicals present in the open environment are DNA mutagens. Nevertheless, few studies have been published on the effect of environmental mutagens on the integrity of extracellular DNA molecules present in complex environments. Biological agents are currently considered the major cause of extracellular DNA degradation. The temporal stabilization of extracellular DNA by transient binding on various surfaces such as clay and sand has recently been reviewed in Nielsen et al. (2006).

Effects of fragmentation on the biological activity of DNA

Endonuclease activity and mechanical shearing lead to shorter DNA fragments. The size of the remaining DNA fragments is crucial to the genetic effects it can produce in exposed bacteria. Studies suggest that biologically significant effects in bacteria result from integration of both short (< 1 kb) and longer fragments (> 10 kb) (Cohan et al., 1991; Feil and Spratt, 2001; Maynard Smith et al., 2000). Longer DNA fragments are more efficient in the transformation process, and can also introduce novel protein producing capacities (Zawadzki and Cohan, 1995). For instance, Carlson et al. (1983) reported that natural transformation of *Pseudomonas stutzeri* occurred most efficiently with chromosomal DNA fragments of approximately 15 kb. Stewart et al. (1991) reported increasing transformation frequencies to rifampicin resistance using longer fragments of up to 3 kb. Integration is also more efficient using longer DNA fragments during natural transformation of *Acinetobacter* sp. (see Fig. 3 in Palmen and Hellingwerf, 1997). A log-linear increase in transformation frequencies was observed when using fragment sizes from 100 bp to 5000 bp (Palmen and Hellingwerf, 1997).

The uptake of DNA of variable size in marine bacteria was examined by Jørgensen and Jacobsen (1996). In contrast to previously described studies, this study reported that uptake of smaller fragments (100 and 250 bp) occurred more rapidly than uptake of larger ones (569 bp). However, they measured cellular absorption of DNA, whereas the other studies (see previous paragraph) determined the genomic integration of functional DNA fragments. After 6 h of incubation, 76% of the 100 bp DNA fragments were taken up and, after 24 h incubation, 52% of the 250 bp DNA fragments and 45% of the 569 bp DNA fragments were incorporated into cell constituents as measured by radioactive labeling of the added DNA. DeFlaun and colleagues have reported that gene-sized fragments of extracellular DNA are present in both freshwater and seawater (DeFlaun and Paul, 1989; DeFlaun et al., 1987). Thus, depending on the sequence length of the genetic trait of interest, DNA fragmentation can lead to a reduced likelihood of uptake in bacteria or to complete genetic inactivation of the DNA. This is interesting in terms of putative spread of GMO-derived DNA, where primarily gene-sized DNA fragments may be relevant. Highly degraded DNA fragments are unlikely to transfer new protein encoding capabilities. However, they may effect allele changes by introducing amino acid substitutions or indels into homologous stretches of a recipient

bacterial genome. Antibiotic resistance marker genes encoding beta-lactamases and aminoglycoside modifying enzymes are generally in the size range of 700–1000 bp. However, additional flanking DNA sequences are necessary to initiate additive integration based on recombination initiated in flanking regions with high DNA similarities. Thus, the minimal DNA fragment size required for horizontal acquisition of chromosomal DNA from GMOs that can produce novel proteins will be sum of the size of the transgene (including regulatory sequences) and the flanking DNA sequences necessary to facilitate homologous recombination based integration events (Bensasson et al., 2004).

LIMITATIONS TO THE STUDY OF THE PERSISTENCE OF DNA

Numerous studies have been conducted on the factors related to the physical persistence and biological activity of environmental DNA. However, these studies are limited to selected environmental conditions (often microcosms), leaving the degradation kinetics of DNA in most open environments unknown. A number of experimental limitations can be identified. The physical methods used to determine the stability of DNA often rely on the prior extraction of total DNA from the environment. The methods applied for DNA extraction vary in terms of the species diversity they yield (Luna et al., 2006). This lack of specificity, together with the efficiency of the applied technique, will ultimately impact subsequent detection and quantification methods (Frostegård et al., 1999; Lindahl and Bakken, 1995; Trevors and van Elsas, 1995). For instance, the level of DNA recovery from soil fluctuates between 8–99% (Robe et al., 2003; Zhou et al., 1996). Moreover, the original sample is often destroyed during DNA extraction, making it difficult to trace its original location and concentration. A loss of signal during sampling, shipping, storage, and processing can occur as well (Martin-Laurent et al., 2001). Residual contamination can further interfere with subsequent analyses, such as PCR and hybridization (Krause et al., 2001; Saano et al., 1993; Tebbe and Vahjen, 1993). The detection limit of DNA isolated from environmental samples amplified by PCR has been reported to vary from 10 to above 10 000 copies (England et al., 1997; 2005; Tsai and Olson, 1992), with each PCR reaction targeting less than 10^7 bacterial genomes per PCR tube (Nielsen and Townsend, 2004). Physical methods that allow *in situ* localization of naked DNA molecules, such as fluorescent *in situ* hybridization, FISH, unfortunately have limited sensitivity in complex environments such as soil, due to the presence of particulate matter and nonspecific binding of probes.

Technological developments for *in situ* detection and quantification of naked DNA, as distinguished from DNA in cells, are needed for the precise localization and quantification of free DNA molecules in complex environments. Such physical assays would be essential in determining the absolute quantities and cycling of environmental DNA and would further require complementary advances in transformation assays to provide information on the availability of free DNA to indigenous bacterial recipients.

CONCLUDING NOTES

Extracellular DNA occurs in all the natural environments studied although rapid microbial degradation of unprotected DNA molecules is expected. The degradation kinetics vary considerably depending on cellular and environmental conditions. Fractions of pure DNA introduced into soil or water can escape immediate degradation and persist for various periods of time (from hours to days). Some of this DNA is sufficiently undamaged that it can transform competent bacteria after re-extraction. DNA fragments from field grown plants have been found for up to several years after cultivation. Thus, current studies suggest that, whereas degradation of the majority of DNA present in various tissues occurs rapidly upon cell death and decomposition, some fragments remain in different environments for prolonged periods of time. Further research efforts should be undertaken to identify and characterize the intra- and extra-cellular processes affecting the release of DNA from various organisms. Such information would reveal the extent and range of exposure to DNA encountered by bacteria in their natural habitats. The need to better understand the environmental fate of extracellular DNA will be further emphasized in biological risk assessment when more novel and artifical recombinant DNA compositions are developed.

Finally, although the implications of extracellular DNA molecules encompassed in the temporal and spatial adaptation and evolution of bacterial species remain unresolved (Nielsen et al., 2007), it should be noted that the mechanistic process of horizontal gene transfer alone does not create an adverse impact. It will be fitness changes of the bacterial transformants caused by the horizontally-transferred DNA that may create an adverse impact that is relevant from a GMO biosafety perspective (Pettersen et al., 2005). Thus, this review has dealt only with one step in the chain of events necessary for an hypothesized adverse effect to materialize.

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