



Biosafety for Large-Scale Containment Level 1 Operations Using Recombinant DNA Technology: No Emerging Hazards

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Abstract

Regulation of technologies using recombinant DNA (rDNA) methods and organisms has eased considerably over the past 30 years. Most of the previous risk assessments focused on the possibility of a recombinant organism or its genetic element establishing itself in the environment if it escaped from a laboratory or cell culture or fermentation facility. Since those assessments were conducted, deliberate releases of recombinant organisms have taken place in various agricultural field trials. This paper reviews the available risk assessments regarding rDNA in the environment and considers the possibility of horizontal gene transfer from eukaryotic cells, released via industrial cell culture or fermentation process waste streams, to prokaryotic cells in the environment.

Introduction

Over the last 30 years, recombinant DNA (rDNA) technology has evolved from an exciting new “ground-breaking” set of techniques into an established science. Industry and public concerns regarding the safety of rDNA technology developed in parallel with the emergence of the science. In the beginning of the rDNA era, the potential of recombinant organisms becoming pathogenic or otherwise causing harm to humans or the environment was an unfamiliar and previously unassessed risk. Now, prokaryotic and eukaryotic expression systems engineered using

rDNA methods are routinely used for the production of a wide range of products for medical, diagnostic, and other uses. The use of biological and physical containment methods, the results from the risk assessment experiments, and a history of safe use of recombinant organisms to date have alleviated the initial concerns regarding the safety of this technology.

This paper reviews the evolution of biosafety practices and details the current industry regulations and approaches for treatment of waste from production operations using rDNA-derived expression systems. Chinese hamster ovary (CHO) cells are referred to throughout as being representative of a recombinant eukaryotic host with an extensive history of safe large-scale use and thus are permissible for use under the U.S. National Institutes of Health’s (NIH) Good Large Scale Practice (GLSP) and the largely equivalent Containment Level 1 regulations in the European Union. The main focus of this paper centers on any possible hazards that might not have been considered extensively to date—namely, the potential for chromosomal DNA from recombinant organisms to cause harm to humans or the environment via horizontal gene transfer to bacteria.

Thirty Years of Risk Assessments for Recombinant DNA Technology

In 1979, Dr. Rollin Hotchkiss wrote to the U.S. NIH Recombinant DNA Advisory Committee: “I did in 1950, after some deliberation, perform the

first drug resistance DNA transformations, and in 1964 and 1965 took part in early warning against indiscriminant 'transformations' that were then being imagined." The extent of scientists' apprehensions regarding this new field of rDNA technology increased and at the June 1973 Gordon Conference, the U.S. NIH was asked to study the issues of safety for laboratory workers. Concern was so great that a number of notable scientists called for a moratorium on rDNA research. These events led directly to the 1975 Asilomar Conference and the creation of NIH oversight of rDNA research in the U.S. Shortly afterwards, Canada, the United Kingdom, and other countries had similar regulations and oversight bodies.

The initial regulations were fairly restrictive, requiring moderate to high physical and biological containment. Risk assessment experiments were initiated to quantify the risks so that performance of risk assessments could be based on data rather than supposition. Risk assessment cannot prove a negative (i.e., that something is impossible). "Risk is a function of the probability of an event and of the scope of its effect" (Lieberman, Israeli, & Fink, 1991; Winkler, 1988). A key question was, "How could the risks associated with this new technology be evaluated?"

Risk Assessments Experiments

One of the worries early in the history of rDNA technology was that a harmless microorganism could become pathogenic. A hybrid *Shigella flexneri*/*E. coli* K-12 was created by cloning of a number of *Shigella* surface antigens into the K-12. Volunteers ingested this recombinant either in a single 3×10^{10} dose or three 3×10^{10} doses, each 1 week apart. None of the volunteers became ill and the K-12 was undetectable after 5 days for those receiving a single dose and within 13 days for those receiving multiple doses (Levine et al., 1977). Other experiments followed, including the cloning of the complete genome of polyoma virus (a mouse oncovirus) into *E. coli* K-12. No infections resulted when mice were injected with high numbers of the recombinant host or the vectors (plasmid and phage) (Israel et al., 1979, 1979a). These and other experiments have led to the conclusion that a nonpathogenic organism cannot become pathogenic from the insertion of a single well-known protein not involved in pathogenicity; moreover,

even multiple pathogen genes will not necessarily transform a nonpathogen into a pathogen (Winkler, 1988; World Health Organization (WHO); 1983).

Another concern was that a person or animal would become colonized with a recombinant organism producing a hormone or other pharmacologically active substance. It was calculated that even if one's gut were completely colonized by *E. coli* K-12, producing insulin, human growth hormone, or interferon and these proteins were not digested, the quantities produced would be too low to have any effect. This theoretical exercise was then experimentally substantiated in animal experiments (Winkler, 1988).

A final concern centered on the possible escape of a recombinant organism and subsequent disturbance of the environment. Adverse environmental impact would require escape, transport to a suitable niche, expression of the recombinant protein, successful multiplication in the face of competition of the indigenous organisms, and then spread resulting in damage due to expression of the rDNA gene (Lieberman, Israeli, & Fink, 1991; Lieberman et al., 1996; Winkler, 1988;). While escape is possible, especially at scales (>10 L) governed by GLSP, the subsequent steps are much less likely to occur. First, the rDNA organisms are leaving a man-made world optimized for growth (i.e., temperature control, nutrients, etc.) and are entering the natural world which has a poorer nutritional status and would very likely be encountering a sudden temperature shift of 10° - 15° C. While organisms can adapt to these conditions, it takes time. During that time, they are being preyed upon and out-manuevered for available nutrients. Unless the rDNA enables the organism to use a substrate that the native population cannot metabolize, or enables the organism to reproduce at a higher rate, it is unlikely that the organism will survive (Lieberman et al., 1996; Winkler, 1988).

Application of the Risk Assessments

Due to many experiments of the type noted above, the lack of injuries to laboratory workers, and the lack of evidence of harm (Organisation for Economic Co-Operation and Development (OECD), 1986; WHO, 1983), the restrictive requirements for working with rDNA have been somewhat relaxed. Certain experiments that required P-2 containment

(now called Containment Level 2/Biosafety Level 2 or BL-2) at the beginning of the rDNA era are now rated as exempt from regulation. One “organism” category affected by this change was mammalian cell lines such as Chinese Hamster Ovary (CHO) cells. It was readily noted that mammalian cells in culture “had no capacity for propagation outside the laboratory” (National Institutes of Health, 1980) and die quickly in the environment, resulting in no environmental risk (Winkler, 1988a). It is now recognized that insertion of a well-characterized gene into organisms with a history of safe use, such as CHO cells, does “not raise any safety considerations beyond those that might be posed by the [gene] products themselves” (OECD, 1986).

It is notable that after about 30 years of rDNA research and industrial use, no harm to the environment, no infections in laboratory workers, and no deaths due to exposure to rDNA organisms have been documented. In contrast, there have been frequent cases of laboratory-acquired illnesses in scientists working with nonrecombinant, pathogenic microorganisms. This does not mean that rDNA technology is without risk. It does mean that the biological and physical containment schemata initiated about 27 years ago have provided protection to laboratory workers and to the environment.

Current Regulatory and Industry Best Practice for Recombinant DNA Waste Treatment

European Community member states implement Directive 98/81/EC through their own legislation governing the contained use of genetically modified microorganisms (GMMs). As might be expected, there is much similarity between the requirements of regulations of member states covering such topics as risk assessment, containment and control, disposal, etc. There are clear edicts on expectations regarding biowaste treatment before disposal. For example, the Genetically Modified Organisms (Contained Use) Regulations of the UK (2000) (Health & Safety Executive, UK, 2000) and Ireland (2001) (Dublin Stationery Office, 2001) both require that GMM-containing waste generated from Con-

tainment Levels 2-4 activities is inactivated by validated means before disposal. The UK regulations state that Containment Level 1 activities (e.g., in Europe, these involve CHO cell lines) must also use validated methods of inactivation while in this category; Irish regulations state it is optional (though in practice expectations are that biopharmaceutical companies will inactivate waste from large-scale production operations).

The well-characterized nature of CHO cell lineages and the over 2 decades of safe use of these cell lines in large-scale (LS) production operations have exempted their categorization in the U.S. from the containment levels BL1-LS to BL3-LS (National Institutes of Health, 2002). The categorization of CHO cell lines under the umbrella of GLSP has resulted in decontamination/inactivation requirements being largely particular to individual state and local government legislation. Hence, certain companies will inactivate CHO cell waste prior to disposal; others will not. Since CHO and other mammalian cells cannot persist in the environment, the only risk factor would be the persistence of their chromosomal DNA and subsequent uptake by competent bacterial cells. Whether the inactivation processes would fragment the DNA sufficiently to disrupt all gene function is outside the scope of this paper. Table 1 compares inactivation practices for large-scale operations using mammalian cells in certain states in the USA and Europe.

Companies implementing inactivation methods are generally expected to use validated procedures. Validation demonstrates that the inactivation method of choice is suitable for the purpose and removes the need to monitor for the GMM in waste streams after the inactivation event during routine operations. For certain classes of operation, usually Containment Level 2 and above, the capability of monitoring for the presence of GMM outside of the contained process is expected under the UK and Irish guidelines mentioned previously. The need for this is dependent on practical feasibility and the risk assessment outcome, and is usually considered on a case-by-case basis. However, for well-characterized Group/Class 1 GMMs such as CHO cell lines used under Containment Level 1 (in Europe), monitoring for the GMM would be expected only under excep-

Table 1

Examples of large-scale biowaste treatments prior to disposal in biotechnology companies currently operating in the USA and Europe as of April 2004. All companies use mammalian cell technology (CHO and/or NS0) for the production of therapeutic proteins. Three different companies in West and East Coast States of the USA are represented. Information gathered through personal experiences and personal communications to the authors. ✓=method used, ✗=method not used.

Company location	No. of inactivation methods prior to disposal	Hypochlorite inactivation	High pH inactivation	Heat inactivation
West Coast, USA. I	1	✗	✗	✓
West Coast, USA. II	1	✗	✗	✓
West Coast, USA. III	1	✓	✗	✗
East Coast, USA. I	0	✗	✗	✗
East Coast, USA. II	0	✗	✗	✗
East Coast, USA. III	1	✗	✓	✗
Mid-West, USA	1	✗	✓	✗
Germany	1	✗	✗	✓
UK	1	✗	✗	✓

tional circumstances (e.g., if there was a serious breach of large-scale process containment).

Horizontal Gene Transfer

A number of factors need to be considered regarding the question of whether eukaryotic genes in the natural environment can transfer to prokaryotic organisms. These factors include natural prokaryotic mechanisms of exchange of genetic elements, and whether prokaryotes have defenses against gene uptake, especially foreign genes. Another consideration is the fate of the DNA in the environment and the stability of the DNA relative to the time required for a competent cell (a cell capable of taking up DNA) to take in the genetic material. Next, one has to consider the fate of the DNA in the prokaryotic cell (i.e., it could be enzymatically digested, circularized into a plasmid, or integrated into the genome), and, finally, whether it would become a functional, expressed gene (Figure 1). Mammalian proteins often

have to undergo posttranslational processing to become a functional protein. Bacteria lack the post-translational machinery to render more complex gene products (glycoproteins) functional.

Eukaryotes and prokaryotes have been coevolving ever since they separated from their ancestral cell an eon ago. During this time, they have been continually exposed to each other's genetic elements. Each year, the decay of pollen, leaves, fruit, and animals results in thousands of tons of DNA being released into the environment (Dale et al., 2002). Marine waters contain 0.2-19 µg DNA/L depending upon where sampled (i.e., coastal, estuarine, or offshore). Freshwater lakes, rivers, and ponds contain from 0.5-25.6 µg DNA/L; freshwater sediment has about 1 mg DNA/g sediment (Lorenz & Wackernagel, 1994); and soils contain in the range of 80-85 µg DNA/g soil (Paget et al., 2002). In order for prokaryotic, and indeed eukaryotic, species to have evolved as separate entities, they have had to develop defenses against incorporating foreign genetic material.

Horizontal Gene Transfer (HGT)

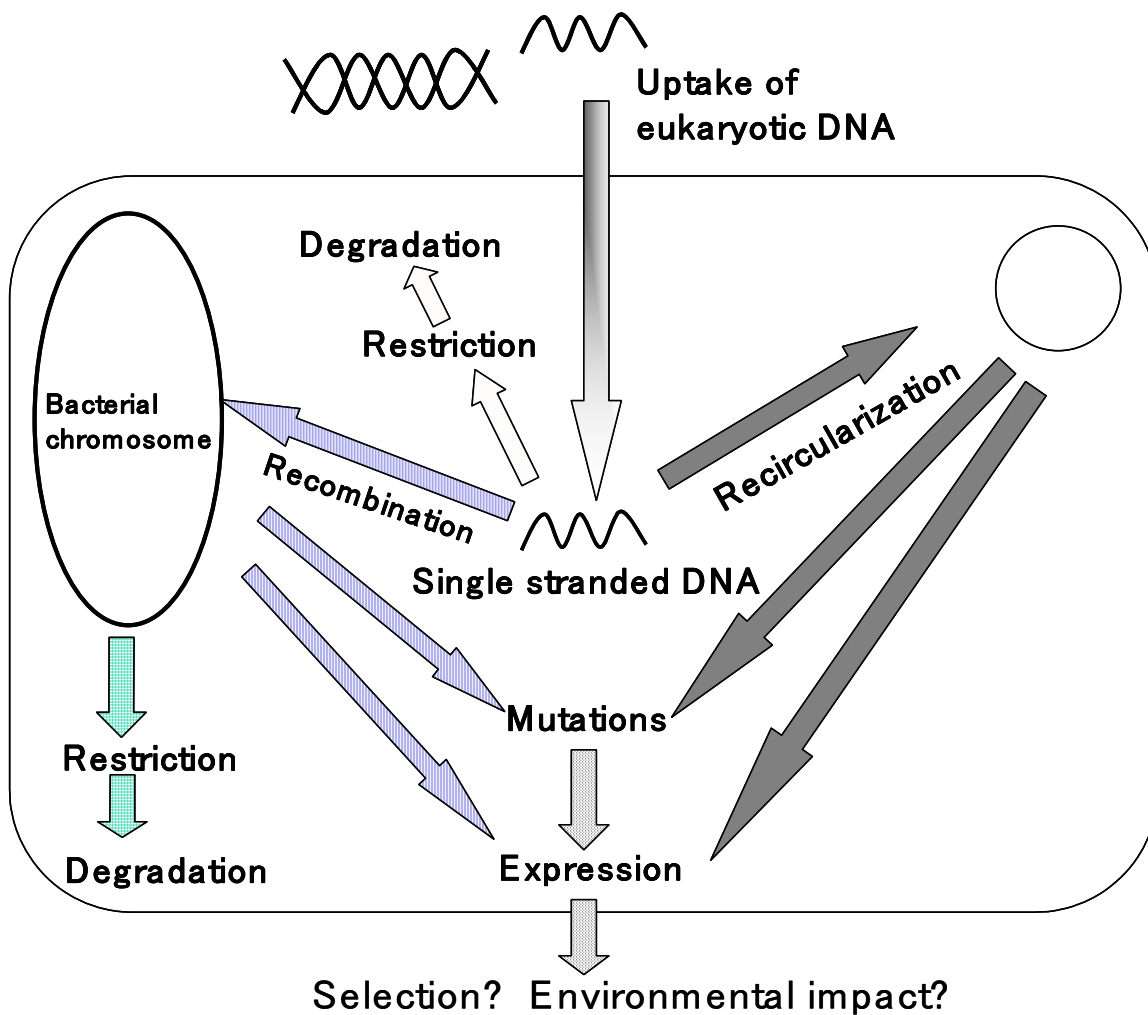
Prokaryotes exchange genes via conjugation (transposons, plasmids), transduction (bacteriophage), and transformation (direct uptake of DNA). Higher eukaryotic organisms, such as hamsters from which CHO cells come, do not have genetic elements such as plasmids or transposons. Viruses that infect eukaryotes do not naturally infect prokaryotes. Even when portions of bacterial plasmids are introduced naturally to plants, there is no reverse transfer of those plasmids back to bacteria. Studies with *Agrobacterium* infection of plants have shown that they transfer to the plants portions of their Ti and Ri plasmids (called T-DNA). The transfer of these plasmids back to bacteria has not been

shown to occur (Nielsen et al., 1998). Thus, the only avenue left for the natural transfer of eukaryotic genes to prokaryotes is via direct gene uptake. For a transfer to take place there has to be a release of functional DNA, persistence of the DNA, and then uptake by a competent cell (Bruns, Reipschlager, Lorenz, & Wackernagel, 1992; Kay et al., 2002). Lastly, in order for the foreign gene to have an environmental effect, the gene must be expressed and translated.

It is widely thought, though not universally, that HGT is a major driving force in the evolution of prokaryotic organisms, playing a role similar to that of sex for eukaryotic organisms (Gogarten, Doolittle, & Lawrence, 2002; Kurtland, Canback, & Berg (2003);

Figure 1

Possible fates of DNA after transformation of a competent bacterial cell.



Nielsen et al., 1998; Ochman, Lawrence, & Grolsman, 2000; Syvanen, 1994; Smith, Feng, & Doolittle, 1992). Mapping the genome of many species, both prokaryotic and eukaryotic, has revealed numerous relationships among organisms. There are difficulties in assigning genetic elements to HGT since genes evolve/mutate at different rates. Thus, the age of a HGT cannot be accurately known. Some HGTs may have occurred close to the splitting of prokaryotes and eukaryotes from the ancestral genera. Some may have been more recent, and some genetic sequences have mutated so much that it will never be known if the gene had entered the cell via HGT.

Barriers to HGT

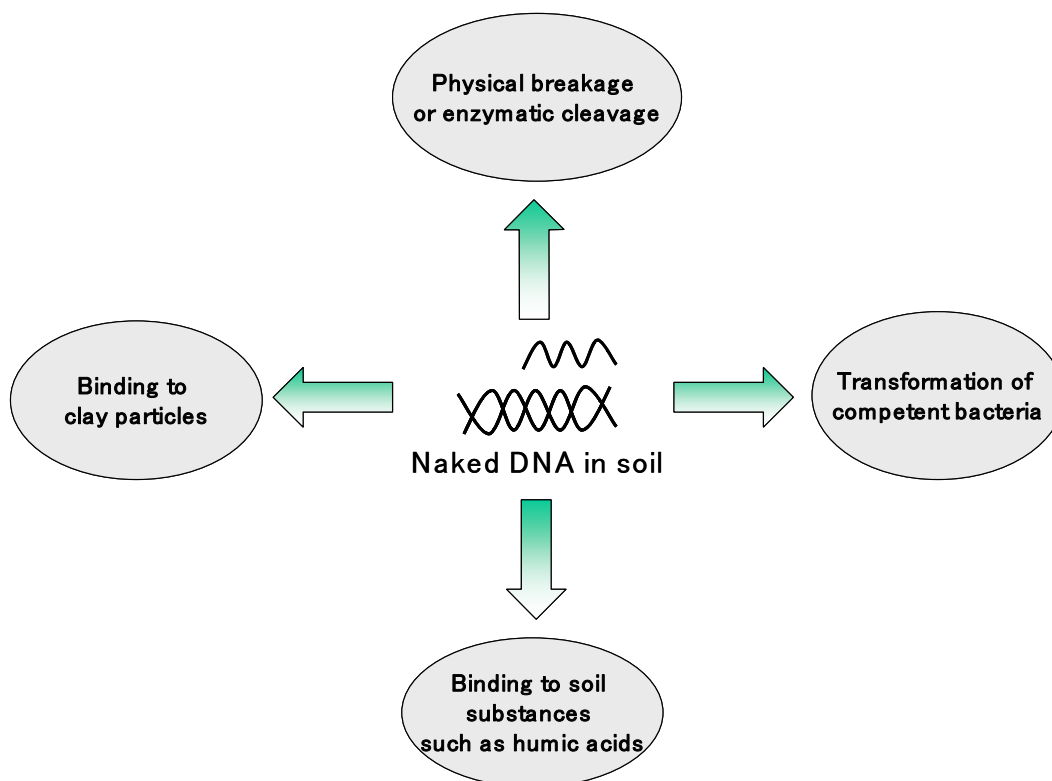
Successful horizontal gene transfer of eukaryotic DNA to prokaryotic species is dependent on the release of free DNA, the persistence of that DNA in the environment, its availability to cells, and its subsequent uptake into cells. As stated earlier, DNA is abundant in the environment. However, it is con-

tinuously under attack by physical shearing forces, chemical modifications, and microbially secreted nucleases and is often not available for transformation since it binds to solids and other matter (Figure 2). Therefore, persistence of chromosomal DNA in the environment does not mean that the DNA is in a form that is available and suitable to transform competent bacteria (Nielsen et al., 1998). Furthermore, most bacteria in the environment are not normally in a competent state and those that are have low transformation efficiencies (Lorenz & Wackernagel, 1994).

Transformation of prokaryotes in the environment can be inhibited by humic acids or enhanced by sand, while clay particles have been reported to either enhance or inhibit transformation depending upon the microcosm (Nielsen et al., 1998). Absence of divalent cations such as Ca^{2+} will prevent bacteria from attaining competency. For example, when Ca^{2+} was below 1 mM, there was no transformation of *E. coli* (Woegerbauer et al., 2002; Lorenz & Wackerna-

Figure 2

Fate of eukaryotic DNA released into the soil environment.



gel, 1994). Additionally, transformation may require temperature shifts. *E. coli* cells were transformed at high efficiency when there was a 0°C to 37°C temperature shift, but a shift from 25°C to 37°C resulted in much lower transformation efficiency, and no transformation occurred at a constant temperature (Woegerbauer et al., 2002). Some bacteria genera, other than *Escherichia*, were capable of being transformed when the temperature was held constant (Lorenz & Wackernagel, 1994). Temperature has a marked effect on transformation in the natural environment. A temperature $\leq 10^\circ\text{C}$ resulted in no detectable transformation *in situ* in a river (Williams et al., 1996).

The size of the DNA fragment also affects transformation. For example, *B. subtilis* and *Ps. stutzeri* required DNA sequences > 1 kb of DNA for transformation (Lorenz & Wackernagel, 1994). Pure cultures of bacteria under laboratory conditions can have very high transformation frequencies, but when *Vibrio* sp. and *Ps. stutzeri* are in their natural, ambient microbial community, their transformation frequency is greatly reduced (Williams et al., 1996). Thus, no one set of environmental conditions will result in all bacteria obtaining competency or having maximal transformation efficiency.

Under laboratory conditions optimized for transformation using bacterial plasmids, transformation of two ubiquitous soil bacteria was completed in 45-120 minutes (Bruns, Reipschlagel, Lorenz, & Wackernagel, 1992). In the natural environment, completion of transformation would probably take longer. The estimated half-lives of DNA in wastewater is 1-13.8 minutes, 4.2-5.5 hours in freshwater, 3.4-83 hours in marine waters depending upon where sampled and the local nutritional status, and 9.1-235 hours in sediments and soils (Lorenz & Wackernagel, 1994; Wackernagel, 1996). Thus, the probability of CHO cell DNA surviving long enough in wastewater to transform bacteria at ambient temperatures is very low.

There are barriers to expression of the transforming DNA if HGT does occur, thus preventing potential environmental impact. These include the failure of DNA stabilization mechanisms within the cell (see above; integration into the bacterial genome by homologous recombination or circularization to

plasmid), the failure of accurate expression of the gene, and posttranslational modification of the gene product. Since single-stranded DNA is usually translocated into the cell during transformation and prokaryotic restriction enzymes attack double-stranded DNA, it is still a matter of debate whether restriction enzyme cleavage is a prevalent resistance mechanism.

The principal barrier to successful transformation and expression is the lack of homology between the foreign DNA and the competent cell's DNA. An exponential decrease in recombination events with an increase in divergence of DNA sequence has been observed with enterobacteria and *Bacillus* spp. (Nielsen et al., 1998). The possibility of genetic exchange from eukaryotes to prokaryotes has been most closely examined during greenhouse and field growth trials of transgenic plants. Clearly, as plants die and decay, their DNA will be released into the soil. Numerous studies have shown that the released DNA is functional and that the DNA in soil can persist from several hours to years depending upon the soil type (Dale et al., 2002; Gebhard & Smalla, 1999; Kay et al., 2002; Paget et al., 1998; Wackernagel, 1996). It is assumed that long-term persistence of DNA enhances the likelihood of the transformation process (Gebhard & Smalla, 1999). The last step in this transformation cascade requires a competent bacterium. In the laboratory, conditions based on temperature shifts, the presence of divalent ions, or electrical pulsing (electroporation) can be easily manipulated to enhance this competence to transformation, but in the natural environment, most of these conditions are not present (Gebhard & Smalla, 1999; Kay et al., 2002; Nielsen et al., 1998). No gene transfer has been found to occur from genetically modified plants to soil bacteria despite the detection of the transgene in the soil (Dale et al., 2002; de Vries et al., 2003; Gebhard & Smalla, 1999; Kay et al., 2002; Nielsen et al., 1998). Only when *Acinetobacter* sp. Strain BD413, a naturally competent cell that is transformable by foreign DNA, was **engineered** to contain a plasmid with homologous sequence to the gene of interest was that eukaryotic gene incorporated (Dale et al., 2002; Gebhard & Smalla, 1999; Kay et al., 2002; Nielsen et al., 1998; Paget et al., 1998).

Extensive review of a variety of bacterial genomes has revealed only six genes that are believed to have been transferred via HGT from eukaryotes to bacteria (Doolittle et al., 1990; Gogarten, Doolittle, & Lawrence, 2002; Nielsen et al., 1998; Syvanen, 1994; Smith, Feng, & Doolittle, 1992). A reanalysis of some of those genes has cast doubt on it being a transformation from a eukaryote to a prokaryote (Doolittle et al., 1990; Smith, Feng & Doolittle, 1992; Syvanen, 1994). Thus, despite the great amount of DNA that prokaryotes are exposed to and the great amount of time that prokaryotes and eukaryotes have coexisted, the incorporation of eukaryotic DNA into bacteria has been a very rare event. In some of these searches, perhaps relationships have not been identified because the fragments transferred are too short to be detected or the genes have undergone rapid sequence alterations that have obscured the origin of the DNA. In addition, detection could have been obscured because the eukaryotic gene transfers have not conferred any selective advantages to the recipient bacteria or because the gene transfers were deleterious to the bacteria and thus the transformants did not persist (Lorenz & Wackernagel, 1994; Nielsen et al., 1998).

Recombinant CHO cell lines are used in the biotechnology industry for the production of many important biopharmaceutical and diagnostic products. Is there a significant risk that naturally occurring prokaryotic organisms in the waste stream or in the waste treatment plant will be transformed when exposed to cell culture waste containing viable CHO cells or their DNA? Consider, for example, a large-scale operation using CHO cells to produce an IgG antibody. A risk assessment of harm or damage resulting from a HGT event would consider the following:

- The genetic information for any eukaryotic product is already in the environment due to death and decay of eukaryotic organisms. It is likely that if this DNA were capable of transforming a prokaryotic organism it already would have occurred.
- Many eukaryotic proteins such as IgG have to be glycosylated and intricately folded to become biologically active, and prokaryotic organisms lack the ability to do this. Thus, even if transformation occurred with a complete coding gene, only an inactive form of these proteins would be produced.

- The barriers associated with HGT and progression to transcription and translation of the genetic information as described above would have to be bypassed. In this hypothetical case, the possibility of homology between the IgG genetic information and the bacterial chromosomal or plasmid DNA is negligible. This lack of homology would apply to a wide range of eukaryotic genetic information.
- For stable carriage of eukaryotic information in a prokaryote, the genetic element would have to assist a bacterium in its competition with other organisms in the environment. As noted previously, this is very unlikely to occur.

The answer to the question of risk posed above is that while we cannot definitively discount all risk, the probabilities of damage due to horizontal transfer of eukaryotic DNA to prokaryotes is vanishingly small and is, thus, a negligible risk.

Conclusion

While there is some evidence that there has been HGT from eukaryotes to prokaryotes, this has been a very rare event. Some of the transfers may have taken place shortly after they had diverged when, theoretically, gene transfers between them would have been easier due to greater homology. There are significant barriers to the transfer of eukaryotic DNA to prokaryotic organisms including, the lack of homology with the recipient's DNA complement and the degradation of DNA in the environment, particularly the rapid degradation in wastewater. Even when DNA is not rapidly degraded, studies searching for gene transfers from genetically modified plants to soil bacteria have shown no transfers of genetic information. Therefore, the probability of genetic transfer of the free recombinant CHO cell gene sequences in process wastewater to bacteria is extremely remote due to (a) the rapid degradation of DNA in wastewater, (b) the low percentage of competent bacteria in the environment, (c) the low transformation efficiency of the competent bacteria that could be present and finally, and (d) the lack of homology between bacterial host DNA and the mammalian cell DNA.

The possibility that there would be an adverse environmental impact is more remote, since even if

the extremely unlikely transformation event occurred, there would have to be expression of the protein and the expression would have to confer a selective advantage. A foreign gene sequence is stable only as long as it conveys a selective advantage. Without the selective pressure, the transferred gene would be lost due to random mutation or deletion (Kurtland, Canback, & Berg, 2003; Lawrence & Ochman, 1998; Ochman, Lawrence, & Grolsman, 2000).

Thus, we conclude that the current best practice of containment as specified by the U.S. NIH and the EU are effective in containing the risks involved in biotechnology development and large-scale production operations using Chinese hamster ovary cells or other cell lines in the Containment Level 1 or GLSP categories. Additionally, the eukaryotic cell-derived DNA in biotechnology facility wastewater poses no known or new risk that warrants monitoring for the DNA itself or for transformation of bacteria in wastewater.

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