



Gene Technology in the Etiology of Drug-resistant Diseases

by Mae-Wan Ho, Terje Traavik, Orjan Olsvik,
Tore Midtvedt, Beatrix Tappeser, C. Vyvyan Howard,
Christine von Weizsäcker and George C. McGavin

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Third World Network

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This is part of a series of papers on biotechnology and biosafety that the Third World Network is publishing with a view to deepening public understanding on the ecological and safety aspects of the new biotechnologies, especially genetic engineering.

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Preface

This study is part of a series of papers on biotechnology and biosafety that the Third World Network is publishing with a view to deepening public understanding on the ecological and safety aspects of the new biotechnologies, especially genetic engineering.

This paper is the result of a report commissioned by the Third World Network and the Ecologist (based in the United Kingdom).

The report has been co-authored by several scientists who examine the potential contribution of genetic engineering biotechnology to the resurgence of infectious drug-resistant diseases.

This is an issue of great public concern since the spread of drug-resistant diseases is rapidly becoming a public health crisis worldwide.

The report makes the following main conclusions:

- According to the 1996 WHO Report, the world is heading for a major crisis in public health as outbreaks of new and re-emerging infectious diseases are striking at increasing frequencies within the past 10 to 15 years. The current strains of pathogens are, moreover, resistant to known treatments; some strains being resistant to all or nearly all drugs and antibiotics. Horizontal gene transfer is now generally recognized to be responsible for the evolution of virulence and the spread of drug and antibiotic resistance. Many patho-

gens have crossed species barriers, having acquired genes from phylogenetically distant species that are involved in their ability to cause disease. Recent findings document the extremely wide scope of horizontal gene transfer and the extensive recombination between genetic material from unrelated species that have contributed to the emergence of virulence and antibiotic resistance.

● The past 15 years coincide with the development of genetic engineering biotechnology on a commercial scale. Genetic engineering depends on designing vectors for cloning and transferring genes and involves artificially recombining and manipulating genes from unrelated species and their viral pathogens, thereby enhancing the probability for horizontal gene transfer and recombination. The urgent question which needs to be addressed is the extent to which genetic engineering biotechnology — by facilitating horizontal gene transfer and recombination — is contributing to the resurgence of infectious, drug-resistant diseases, and will continue to do so if allowed to proceed unchecked.

● An enquiry into the possible contribution of genetic engineering biotechnology to the etiology of infectious diseases is all the more pressing in the light of other relevant recent findings indicating that microorganisms genetically engineered for “contained use” may not be effectively contained. Thus, biologically “crippled” strains of bacteria can survive in the environment to exchange genes with other species; DNA released from cells is not readily broken down in the environment, thereby retaining the ability to transform organisms; some viral DNA can be more infectious than the virus itself; and routine chemical treatments for inactivating pathogenic microorganisms and viruses, before they are discharged into the environment, may be ineffective, leaving a substantial percentage of pathogens in an active infectious state. The combination of the different kinds of evidence is sufficiently compelling, especially in view of the precautionary principle, to warrant, at the very least, an independent public enquiry into genetic engineering biotechnology as a factor in the etiology of infectious diseases.

The Third World Network would like to thank the authors of this report for their contribution to the scientific inquiry and discussion of this highly important subject.

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Executive Summary

The world is heading for a major crisis in public health with the escalated resurgence of drug- and antibiotic-resistant infectious diseases since commercial-scale genetic engineering biotechnology began in the early 1980s. Some of the more recent examples:

- Infectious diseases were responsible for one third of the 52 million deaths from all causes in 1995
- Salmonella infections have gone up 20-fold in some European countries since 1980
- *Escherichia coli* 0157:H7 infections were up 10-fold in England and Wales and 100-fold in Scotland between 1986 and 1996
- Vancomycin resistance rose from 3% to 95% in San Francisco hospitals between 1993 and 1997
- A *Staphylococcus* strain invulnerable to all known treatments arose in 1997
- Fifty new viral pathogens attacking humans emerged between 1988 and 1996

[Note: Since this manuscript went to press, there have been further rapid developments in antibiotic resistance. Strains of three other dangerous bacteria apart from *Staphylococcus aureus* are now in-

vulnerable to *all* known antibiotics: *Enterococcus faecalis* (blood poisoning and wound infection) *Pseudomonas aeruginosa* (blood poisoning and pneumonia) and *Mycobacterium tuberculosis* (TB) (Dobson, R. (1998). Rise of superbugs linked to antibiotics in animal feed. *Independent on Sunday*, 3 May, 1998).]

The evolution of virulence and the spread of drug and antibiotic resistance are now linked to the extensive horizontal gene transfer and recombination events among bacteria and viruses, many of which may have occurred in recent years:

- Unrelated species have identical virulence and antibiotic resistance genes
- A new *Vibrio cholerae* strain in the 1992 Indian epidemic was generated by horizontal gene transfer and recombination
- *E. coli* 0157:H7 arose by horizontal gene transfer of Shiga-toxin from *Shigella*
- Multiple-antibiotic resistance is found to be generated by horizontal gene transfer and recombination

The major orientation of genetic engineering is to enhance horizontal gene transfer and to break down species barriers by means of artificial vectors — chimaeric combinations which do not occur in nature. These are made from a combination of disease-causing viruses, and plasmids and transposons which carry and spread virulence and antibiotic resistance genes.

These effects — including the spread of antibiotic resistance genes — are further enhanced by the profligate use of antibiotics in biotechnology, farming and medicine:

- Artificial vectors have increased host ranges and increased the tendency for horizontal gene transfer and recombination

- Artificial vectors contain antibiotic resistance genes as selectable markers which are often also present in the transgenic organism
- Antibiotics increase the frequency of horizontal gene transfer 10- to 10,000-fold

The first genetic engineers called for a moratorium in the Asilomar Declaration of 1974. They were concerned that new viral and bacterial pathogens could be generated inadvertently by genetic manipulation. Their concern is justified given how virulence is easily evolved and shared:

- Virulence genes exist in blocks, carried by highly chimaeric bacterial viruses that have undergone numerous recombination events
- Many virulence genes are targeted at eukaryotic functions, and may have been acquired from eukaryotic cells by horizontal gene transfer and recombination
- Unrelated genera of pathogens share nearly identical blocks of virulence genes
- Non-pathogens are converted into pathogens in single, quantum transfers of blocks of virulence genes

Now the fears of these early genetic engineers may have been realized, as subsequent guidelines set up to regulate genetic engineering biotechnology are far from adequate. For example, legal limits of “tolerated releases” from contained use vastly exceed the minimum infective dose of some pathogens: 10,000 colony-forming units/ml in air or water versus a minimum infective dose of 50 bacteria for *E. coli* 0157:H7.

These regulatory guidelines are based largely on untested assump-

tions which have been invalidated by recent scientific findings, such as the following:

- Biologically “crippled” laboratory strains of bacteria can often survive in the environment to exchange genes with other organisms
- Routine chemical inactivation methods may leave up to 10% of viruses and other pathogens in an infective state
- DNA released from dead and living cells persists in the environment and is transferred to other organisms
- Naked viral and vector DNA may be more infectious, and have a wider host range than the virus
- Vector DNA resists digestion in the gut of mice, enters the bloodstream to infect white blood cells, spleen and liver cells, and is integrated into the mouse cell genome

In the meanwhile, large amounts of hazardous transgenic micro-organisms and recombinant DNA have been and continue to be released into the environment. We may only be seeing the tip of the iceberg as far as the contribution of genetic engineering biotechnology to the etiology of infectious diseases is concerned. There is an urgent need to assess this factor and to tighten existing regulations. Instead of which, the European Union is currently attempting to relax the guidelines on both deliberate release and contained use of GMOs. This is an irresponsible move in the light of current scientific knowledge.

Chapter 1

The World Health Crisis

The world is heading for a public health crisis. At least 30 new diseases such as AIDS, Ebola, several kinds of hepatitis and other deadly viruses have emerged over the past 20 years (see Table 1, compiled from WHO Report 1996 [1] and other sources [2]), while old infectious diseases such as tuberculosis, cholera, malaria and diphtheria are coming back worldwide. Practically all the pathogens are resistant to drug treatment, many to multiple antibiotics. In 1995 one third of the 52 million deaths from all causes in the world were due to infectious diseases; over half of these in young children. The top killers were tuberculosis (3.1 million, mostly adults), malaria (2.1 million, including 1 million children), hepatitis B (1.1 million) and AIDS (more than 1 million). "The optimism of a relatively few years ago that many of these diseases could easily be brought under control has led to a fatal complacency among the international community," says the Director-General of the World Health Organization, Dr. Hiroshi Nakajima, in a press release.

Emerging diseases have become a major public health concern during the 1990s.

Table 1. New diseases within the past 20 years (incomplete list)

1977: <i>Legionella pneumophila</i> , potentially fatal Legionnaires' disease
1977: Ebola virus, haemorrhagic fever — fatal in up to 80% of cases.

- 1977: Hantaan virus, potentially fatal haemorrhagic fever with renal syndrome.
- 1977: *Campylobacter jejuni*, diarrhoea.
- 1980: Human T-lymphotropic virus I (HTLV-1), T-cell lymphoma-leukemia
- 1981: *Staphylococcus aureus*, bacteria, toxic shock syndrome
- 1982: *Escherichia coli* O157:H7 bacteria, bloody diarrhoea
- 1982: HTLV-2 virus, hairy-cell leukaemia
- 1983: *Helicobacter pylori*, bacteria, peptic ulcer disease and stomach cancer.
- 1983: Human immunodeficiency virus (HIV), AIDS.
- 1985: *Enterocytozoon bieneusi*, parasite, persistent diarrhoea
- 1986: *Cyclospora cayentanensis*, parasite, persistent diarrhoea
- 1988: Hepatitis E virus, epidemics of jaundice in hot climates
- 1988: Human herpesvirus 6, fever and rash
- 1988: *Salmonella typhimurium*, bacteria, diarrhoea
- 1989: Hepatitis C virus, which causes liver cancer as well as liver disease.
- 1989: *Ehrlichia chaffeensis*, bacteria, infection
- 1991: Guanarito virus, Venezuelan haemorrhagic fever.
- 1991: Encephalitozoon hellem, parasite, conjunctivitis
- 1991: New species of Babesia, parasite, infection
- 1992: New strain Vibrio cholerae O139, epidemic cholera.
- 1992: *Bartonella henselae*, bacteria, cat-scratch disease, *Bacillus angiomatosis*
- 1993: Sin Nombre virus, adult respiratory distress syndrome
- 1993: *Encephalitozoon cuniculi*, parasite, disseminated disease
- 1994: Sabiá virus, which causes Brazilian haemorrhagic fever
- 1994: Equine morbillivirus, respiratory illness
- 1995: Human herpesvirus 8, Kaposi's sarcoma in AIDS patients.
- 1995: New monkeypox virus, human-to-human transmission, potentially fatal
- 1996: New rabies in Australia
- 1996: New hantavirus, human-to-human transmission
-

1.1 Has the incidence of infectious diseases and drug resistance gone up recently?

Precise epidemiological data are not yet available, but the signs are that both the incidence and severity of outbreaks of multidrug-resistant pathogens may have experienced a sharp upturn within the past 10 to 15 years. For example, *Salmonella* infections have gone up 20-fold in some countries in Europe since 1980 [3]. Similar increases are reported for haemorrhagic *E. coli* 0157 food poisoning: between 1986 and 1996, the frequency of infection increased 10-fold in England and Wales, and 100-fold in Scotland [4].

The first widely used antimalarial drug, chloroquine, came into use during World War II, and resistance did not appear until the early 1960s. By contrast, the new drug mefloquine, released in 1985, became useless in 60% of malaria cases within five years [5]. Comparable accelerations in the development of antibiotic resistances took place in the same period. Antibiotics were introduced in the early 1940s to treat infectious diseases, and resistance did not appear until the early 1950s [6]. Resistance to penicillin, ampicillin and antipseudomonas penicillins in *Staphylococcus aureus* went from almost 0% in 1952 to more than 95% in 1992 [7]. By the 1980s, *S. aureus* had also developed high levels of resistance to the synthetic penicillin, methicillin and all other β -lactams. The new fluoroquinolone antimicrobial, ciprofloxacin, was introduced in the mid-1980s, but resistance to it had reached more than 80% by 1992. A study carried out by the Centers for Disease Control showed that ciprofloxacin resistance in *S. aureus* went from less than 5% to greater than 80% within one year [7].

By 1990, nearly every common pathogenic bacterial species had developed varying degrees of antibiotic resistance, often, *multiple* resistances. These include, besides *Staphylococcus aureus* (toxic shock syndrome, postoperative infections), *Streptococcus aureus* (toxic

shock-like syndrome), *S. pneumoniae* (pneumonia), *S. pyogenes* (rheumatic fever), *Haemophilus influenzae* (meningitis), *Mycobacterium leprae* (leprosy), *Neisseria gonorrhoeae* (gonorrhea), *Shigella dysenteriae* (dysentery), and several other species of microbes that infect the human gut: *E. coli*, *Klebsiella*, *Proteus*, *Salmonella*, *Serratia marcescens*, *Pseudomonas*, *Enterococcus faecium*, *Enterobacteriaceae* and *Vibrio cholerae* (cholera) [6]. A strain of *Staphylococcus* isolated in Australia was resistant to 31 different drugs including cadmium, penicillin, kanamycin, neomycin, streptomycin, tetracycline and trimethyloprim [8].

The various resistance capabilities were due to genes carried on different plasmids (independently replicating units of genetic material) that could be separately passed on from one bacterium to another. Two strains of *E. coli* isolated in a transplant ward outside Cambridge were resistant to 21 out of 22 common antibiotics: imipenem, as well as cefotaxime, ceftazidime, ciprofloxacin, gentamicin, ampicillin, azlocillin, coamoxiclav, timentin, cephalixin, cefuroxime, cefotaxime, cefamandole, streptomycin, neomycin, kanamycin, tobramycin, trimethoprim, sulfamethoxazole, chloramphenicol and nitrofurantoin [9]. These multi-resistant strains of bacteria are rapidly becoming totally invulnerable to treatment. Scientists in Japan have already isolated a strain of *Staphylococcus aureus* that is resistant even to the last-resort antibiotic, vancomycin [10]. Recent data showed that vancomycin resistance in enterococci grew from 3% in 1993 to 95% in 1997 in hospitals in San Francisco [11]. In Italy, erythromycin resistance in *Streptococcus* increased 20-fold just between 1993 and 1995 [12].

1.2 Factors contributing to the recent resurgence of infectious diseases

The precise reasons for the resurgence of infectious diseases since the 1980s are not known. Many contributing factors have been suggested [1], among them,

- population growth combined with rapid urbanization,

- wars, civil turmoil and natural disasters,
- rapid increase in international air travel and the growing traffic in trade,
- expanding areas of human habitation and the consequent environmental destruction,
- social changes including the clustering of young children in day-care centres and growing numbers of the elderly in nursing homes,
- complacency towards infectious diseases in the public health sector and collapse of public health systems due to economic or social crises,
- the overuse and abuse of antibiotics in intensive farming and medicine.

One factor which has not yet been considered is genetic engineering biotechnology.

1.3 Gene technology and gene ecology

Gene ecology is an emerging discipline. It is prompted by recent findings that the genetic material — DNA or in some instances, RNA — can transfer *horizontally* from one organism to another through the external environment, instead of *vertically* by reproduction. Gene ecology is the totality of how genes function, mutate, move, transfer and recombine, subject to feedback regulation from the interconnected levels of the genome, the physiology of the organism and the external ecological conditions.

Gene technology, or genetic engineering, can profoundly disturb the ecology of genes. The past 15-20 years witnessed the rise of genetic engineering biotechnology on a commercial scale. It is a technology for manipulating and transferring genes horizontally between species that do not normally interbreed. It is designed to break down species barriers and, increasingly, to overcome the species' defence mechanisms which degrade or inactivate foreign genes [13, 14].

For the purpose of manipulating, replicating and transferring genes, a range of artificial vectors have been constructed. Natural vectors for horizontal gene transfer comprise replicating, often mobile units of genetic material: viruses, plasmids and transposons. However, other pieces of DNA can also be taken up by cells and hence act as agents for horizontal gene transfer. Viruses are infectious particles consisting of genetic material wrapped in a protein coat. Animal and plant viruses cause many diseases including cancer; viruses that attack bacteria are bacteriophages or phages for short. Plasmids are replicating units (replicons) of genetic material outside the chromosome. Transposons are mobile replicating units (jumping genes) that can move from one site to another in the same or different chromosome, or from chromosome to plasmid and *vice versa*. Plasmids and transposons typically carry virulence genes and genes for antibiotic resistance.

Artificial vectors are made by joining together parts of the most infectious natural vectors in order to enhance horizontal gene transfer. The artificial vectors are “crippled”, which means that most, if not all, of the genes causing disease and infectivity are removed. *But* it does not mean that those functions cannot be supplied by, or regained from, other viruses and parasitic genetic agents that are always present in the environment and in the cells of all organisms. The gene to be transferred is as a rule integrated into the genetic material of the vector; viruses, however, can also transfer genes that are not integrated, but merely packaged within the protein coat (Fig. 1).

To make a transgenic organism, the vector carrying the foreign gene to be transferred — transgene — is allowed to infect the cells of the organism. Once inside the cell, the vector can either multiply many copies of itself or become integrated into the genome of the cell (Fig. 2).

Most artificial vectors possess one or more antibiotic resistance

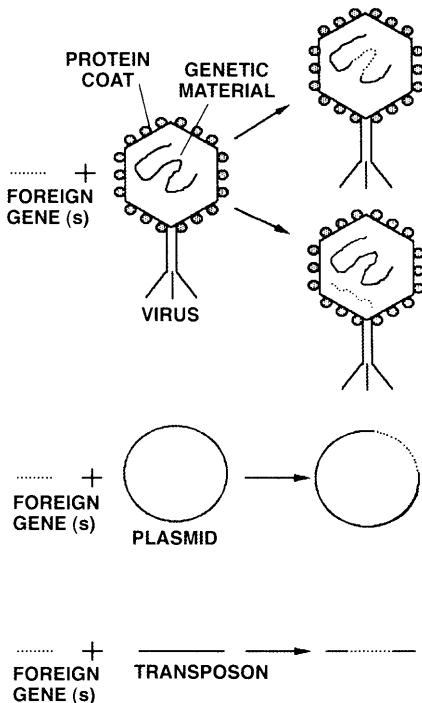


Figure 1. How vectors can transfer genes. The gene(s) to be transferred (dotted line) are usually integrated into the genetic material of the vector; viruses can also transfer genes that are not integrated, but merely packaged within the protein coat.

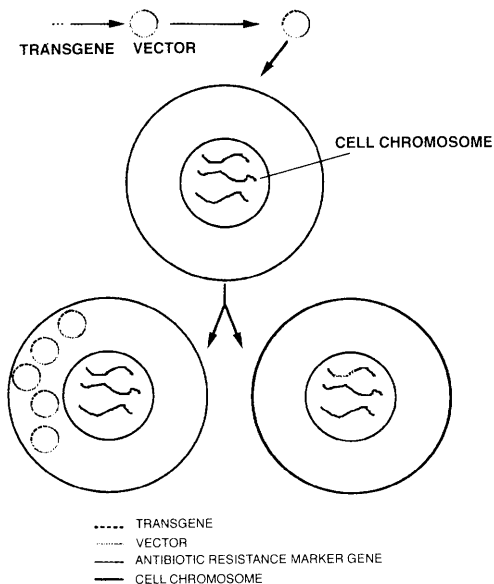


Figure 2. **Genetic engineering makes use of artificial vectors for replicating and transferring genes.** The gene to be transferred (transgene) is inserted into a vector containing one or more antibiotic resistance marker gene(s), which makes it possible to select for cells that have taken up the vector carrying the transgene. The vector carrying the transgene and marker gene(s) can either replicate many copies of itself in the cell or become integrated into the genome of the cell.

marker genes. This is because the movement of the gene(s) transferred can be tracked by selecting with the appropriate antibiotic(s).

There are three main routes for horizontal gene transfer: infection with viruses (transduction), through pieces of genetic material taken up into the cell from the environment (transformation); or by unusual mating taking place between unrelated species (conjugation). As the entire orientation of genetic engineering is to facilitate horizontal gene transfer, it is to be expected that antibiotic resistance genes as well as virulence genes will inadvertently spread and recombine to generate new drug- and antibiotic-resistant pathogens.

Indeed, the evolution of virulence and the spread of drug and antibiotic resistance are now linked to the extensive horizontal gene transfer and recombination events among bacteria and viruses, many of which may have occurred in recent years. For example, horizontal gene transfer and subsequent genetic recombination have given rise to the new bacterial strains responsible for the cholera outbreak in India in 1992 [15-17], and for the *Streptococcus* epidemic in Tayside in 1993 [18-21]. The *E. coli* 0157:H7 strain involved in the recent outbreaks in Scotland is believed to have originated from horizontal gene transfer from the pathogen, *Shigella* [22]. Many unrelated bacterial pathogens, causing diseases from bubonic plague to tree blight, are now found to share an entire set of genes for invading cells, which have spread widely by horizontal gene transfer [23]. Similarly, genes for antibiotic resistances have been transferred horizontally and have recombined with one another to generate multiple antibiotic resistances throughout the bacterial populations [24]. Antibiotic resistance genes spread readily between human beings, and from bacteria inhabiting the gut of farm animals to those in human beings [25]. Antibiotic-resistant strains of pathogens have been endemic in many hospitals for years. In the USA, up to 60% of hospital-acquired infections are antibiotic resistant [1]. A special form of multiple resistance, which confers resistance to a wide range of chemically unrelated drugs, made its appearance among pathogens in the early 1990s (see section 3.2), al-

though it has been produced in *E. coli* in the laboratory since the 1980s [26].

Even more disturbing is the finding that antibiotics can increase the frequency of horizontal gene transfers 10- to 10,000-fold [27-30]. Thus, antibiotics create the very conditions facilitating the spread of antibiotic resistance. The evolution and spread of antibiotic resistance predate genetic engineering, and are largely due to the profligate use of antibiotics in intensive farming, and in medicine [31, 32]. However, the abuse of antibiotics *per se* does not account for the emergence of new viruses and new virulent strains of bacteria. Fifty new viruses were identified just between 1988 and 1996 [33]. *The urgent question which needs to be addressed is the extent to which genetic engineering biotechnology — by facilitating horizontal gene transfer and recombination — is contributing to the resurgence of infectious, drug-resistant diseases, and will continue to do so if allowed to proceed unchecked.*

This question is all the more important in the light of yet other recent findings indicating that microorganisms genetically engineered for “contained use” may not be effectively contained, *while our regulators are contemplating further relaxations of the guidelines on contained use.* Thus, biologically “crippled” strains of bacteria have actually been found to survive in the environment to exchange genes with other species (reviewed in [34, 35]). Naked viral DNA

* The UK Health and Safety Executive, on the recommendation of the Advisory Committee on Genetic Modification (ACGM), produced a consultation document, Draft Guidance on Certificate of Exemption No. 1, which would allow commercial and other contained users to release certain classes of live genetically engineered microorganisms (GEMs) directly into the environment as liquid wastes on notification, without the need to monitor for the survival and subsequent evolution of the GEMs.

may be more infectious than the virus itself [36]. DNA released from cells is not readily broken down in the environment, thereby retaining the ability to transform organisms (reviewed in [34, 37, 38]); and routine chemical treatments for inactivating pathogenic microorganisms and viruses, before they are discharged into the environment, may be ineffective, leaving a large percentage of pathogens in an active, infectious state [39]. The combination of evidence is sufficiently compelling, especially in view of the precautionary principle, to warrant, at the very least, an independent public enquiry into the possible contributions of the industry to the etiology of infectious diseases.

This Report will review the following topics:

- (a) The origins and nature of antibiotic resistance and its spread by horizontal gene transfer and recombination.
- (b) The mechanisms and scope of horizontal gene transfer.
- (c) The evolution of virulence and its spread by horizontal gene transfer.
- (d) Genetic engineering and horizontal gene transfer.
- (e) Other recent findings relevant to the possible contribution of genetic engineering biotechnology to the etiology of infectious diseases.

On account of the enormity of the subject areas, it is impossible for us to be exhaustive in our review. Our goal is to supply enough information for readers to judge for themselves whether the strength of the evidence together with the precautionary principle will justify the public enquiry proposed.

Chapter 2

Antibiotics and Emerging Pathogens

When antibiotics were first introduced, microbial geneticists did not believe antibiotic resistance would pose any problems, based on the very low spontaneous mutation rates observed [24]. Mutation — random changes in the genetic material — and selection — survival of the fittest — were thought, by most biologists, to sufficiently explain all characteristics of organisms. If the mutation rates are very low, then “selection pressure” would have to be very strong for the character to spread.

Soon after antibiotics were used in medicine, it was discovered that livestock lived longer and grew faster when they were fed antibiotics to overcome infections. This led to “prophylactic” treatments. In fact, antibiotics were routinely fed to chickens, cattle, pigs and dairy cows to promote growth. They also extended the shelf-life of meat, poultry, eggs and dairy products. In the 1970s, it was discovered that chickens given high doses of antibiotics resulted in resistant *Salmonella* strains being detected in both the meat and the eggs. By 1983, mutant strains of antibiotic-resistant *Salmonella* that attacked human beings had emerged [6]. The emergence of new pathogens is strongly associated with mutant strains that cross species barriers.

One of the clearest and most disturbing examples of mutant bacteria crossing from domestic animals to human beings, and of the transformation of a previously benign strain into a pathogen, is *Escherichia coli*, a common bacterium inhabiting the intestine of all

human beings and many other mammalian species. Most of the time, *E. coli* is harmless, which is why it has been the major tool for genetic engineers, who have used it and its plasmids routinely to clone genes since the 1970s [40]. *E. coli* is the most intensively manipulated organism, and genes from species in every Kingdom have been transferred to and *cloned* in it. Cloning means multiplying identical copies, and can refer to a gene as well as an organism with identical genetic make-up. Perhaps it is not surprising that *E. coli* has emerged as a major pathogen.

The most prominent among pathogenic strains, *E. coli* 0157:H7, appeared in 1982, which, far from being benign, caused dangerous haemorrhages of the colon, bowel and kidneys of human beings; and it broke out suddenly and simultaneously in several states in the USA [41]. Like most *E. coli* strains in the 1980s, it was moderately resistant to the antibiotics ampicillin and tetracycline. Since then, many outbreaks have occurred all over the world, and in increasing frequencies. A mass outbreak in Japan in 1996 affected 10,000, with 12 deaths in children. A series of outbreaks in Scotland in 1997 claimed 20 lives and made hundreds ill [42, 43].

Genetic fingerprinting — a technique that fragments the DNA using specific enzymes and reading the fragments — backs up epidemiological evidence that *E. coli* 0157:H7 arose recently; and up to 1993, was endemic only to cattle in USA, Canada and Great Britain, and absent in cattle in the Far East (Thailand) [44]. *E. coli* mutates fairly rapidly, hence the low degree of genetic variation observed among the 0157:H7 isolates before 1993 suggests they originated recently from a single clone. Most cases came from contaminated meat. However, outbreaks have also been associated with cheese, salami, raw vegetables, unpasteurized apple juice and water [46]. The *E. coli* 0157, like other water-borne pathogens such as *Cryptosporidium*, *Giardia* and *Shigella*, had developed resistance to chlorine [6, p. 430]. The mass outbreaks in Japan in 1996 were thought to be linked to white radishes [43], which may have come from contaminated soil. The specific toxicity of the strain is attrib-

uted to the Shiga-like toxin genes acquired by horizontal gene transfer from *Shigella* [22, 46]. One of these genes, *VT1*, is almost completely identical in base sequence to that of *Shigella*, indicating again, that the gene transfer was a very recent event.

Studies of dozens of emergent species of pathogens showed that genes for antibiotic resistance and virulence often reside in the same regions of the bacterial DNA, in plasmids or transposons which may be passed horizontally from one species to another, picking up extra genes, recombining and generating novel combinations in the process [6, 24].

2.1 A profusion of mechanisms and genes for antibiotic resistance

A profusion of biochemical mechanisms and genes are involved in antibiotic resistances, fully matching, if not surpassing the variety of targets at which antibiotics are aimed [7, 24, 46-48]. Most antibiotics have a single primary target, usually a single step in the synthesis of macromolecules essential to the bacterial cell, and preferably do not affect their eukaryotic host cells. Thus several classes of antibiotics are aimed at different steps in synthesizing the bacterial cell wall. These include the penicillins or β -lactams, vancomycin and ristocetin, D-cycloserine, fosfomycin and bacitracin. Other classes of antibiotics are aimed at bacterial enzymes involved in the synthesis of DNA, RNA and proteins. These enzymes differ from their eukaryotic counterparts, although the effects of the antibiotics may not always be so selective, and are accompanied by varying degrees of toxicity for the eukaryotic host. Metronidazole, quinolones, nalidixic acid and novobiocin target DNA and DNA synthesis, while actinomycin and rifamycin inhibit transcription (RNA synthesis). Translation (protein synthesis) is targeted by a wide variety of antibiotics: streptomycin and other aminoglycosides such as kanamycin, neomycin, gentamicin, tobramycin, amikacin and netilmicin; tetracyclines, puromycin, chloramphenicol, eryth-

romycin, lincomycin and clindamycin, and fusidic acid. In addition, antimetabolites such as sulfonamides and trimethoprim inhibit the synthesis of essential metabolites.

Resistances to every class of antibiotics have been identified (see Table 2, modified from [24]), the genes responsible may be found on the chromosome or in plasmids.

Table 2. A summary of mechanisms for antibiotic resistance

Mechanism	Antibiotic
1. Modification of target to reduce or eliminate binding of antibiotic	β -lactams Erythromycin Lincomycin Streptomycin
2. Inactivation of antibiotic by enzyme	β -lactams
a. Hydrolysis	Erythromycin
b. Derivatization	Aminoglycosides Chloramphenicol Fosfomycin Lincomycin
3. Sequestration of antibiotic by protein binding	β -lactams Fusidic acid
4. Metabolic bypass	Sulfonamides Trimethoprim
5. Binding of antibiotic by specific immunity protein	Bleomycin
6. Overproduction of antibiotic target	Sulfonamides Trimethoprim

7. Reduced uptake	Chloramphenicol
8. Active efflux from cell	Tetracycline Chloramphenicol Multiple resistance to broad ranges of antibiotics

One of the commonest mechanism of resistance is the degradation or modification of the antibiotic by specific enzymes. Thus, the β -lactams are degraded by lactamases, all of which have arisen from one ancestral gene. On the other hand, aminoglycosides are inactivated by specific modifying enzymes encoded by 30 or more different genes, while chloramphenicol is inactivated by many acetyltransferases encoded by at least a dozen genes. Mutation of the specific antibiotic target is also frequently involved in resistances. For example, streptomycin is overcome in *Mycobacterium* by mutations in the ribosomal RNA (rRNA) molecules required for protein synthesis, so that they have reduced affinity for streptomycin. Antibiotics may be inactivated simply by sequestration, or binding permanently to specific proteins. A metabolic bypass may appear to a reaction blocked by the antibiotic, or the inhibited protein may simply be overproduced by the bacteria in order to overcome the inhibition. Finally, resistance may be conferred by reduced uptake of the antibiotics or by increased rates of pumping them out of the cell — active efflux mechanisms.

Active efflux mechanisms are especially important for the multiple resistances to broad ranges of structurally unrelated antibiotics and drugs that have appeared in pathogenic bacteria within the past 10 years. These will be dealt with in section 3.2.

Quite often, several biochemical mechanisms are involved in giving protection against a single antibiotic, resulting in a very high level of resistance. Cross-resistances are also common within a class

of antibiotics such as the aminoglycosides — so at least one kanamycin resistance gene used as a genetic marker has been found to confer cross-resistance to two new-generation aminoglycosides, amikacin and tobramycin [49]. A recently documented case of antibiotic cross-resistance is even more serious, as *Enterococcus faecium* inhabiting the gut of turkeys on farms using avoparcin as growth promotor is found to have developed cross-resistances to vancomycin and teicoplanin [50], the last defence against multidrug-resistant *Staphylococcus aureus*.

Where do the genes conferring antibiotic resistances come from? And how do they manage to spread so quickly? Let us first address the question of the origin of these genes.

Chapter 3

Origins of Antibiotic Resistance Genes

3.1 Biochemically specific mechanisms

There are several sources of antibiotic resistance genes (reviewed by Davies, [24]). The first is from organisms producing the natural antibiotic, such as penicillin, which also produce the inactivating enzyme. As new β -lactams are introduced, the genes encoding the β -lactamases undergo mutational changes, often involving only one base substitution and a single amino-acid difference in the enzyme. That would be sufficient to enable the enzyme to recognize the new drug and to break it down. In this way, the enzyme extends its spectrum of resistances to new drugs. Numerous mutational variants of β -lactamase have already been identified, showing the ease with which appropriate mutational changes can arise in this gene. The variants derive from different genes which are themselves traced to a single ancestral gene. The versatility of the β -lactamase gene is such that when a series of inhibitors were made against the enzyme and used in combination with the β -lactam antibiotic — a kind of belt-and-braces approach — mutant enzymes arose, which not only broke down the new antibiotic, but were, at the same time, insensitive to the inhibitor.

More often, however, it is not at all clear where the antibiotic resistance genes come from. New genes seem to appear from nowhere. For example, the 30 or more genes involved in resistances to different aminoglycosides are not related to one another. Some are thought to have arisen from mutations of pre-existing genes

encoding enzymes catalyzing normal metabolic reactions in the cell. Others may involve activation of previously “cryptic” or hidden genes in the bacterial genome (see section 3.2 below). Or they may have been acquired by gene transfer from another bacterium.

3.2 Active efflux mechanisms

Active efflux mechanisms are due to 20 or more genes in at least four gene families (see Table A.1 in Appendix I, modified from [47]), members of which can be found on plasmids or on the chromosomes. The genes code for transporter proteins situated across the cell membrane that pump substances out of the cell, using ATP (adenosine triphosphate) or protons as the source of energy. Recently, it has been shown that the genes for several efflux pumps are modulated in response to environmental conditions, and that many of the systems are also inducible by the substances to which they give resistance.

The chromosomally encoded efflux genes in *E. coli* belong to one or another of four inducible operons: *marRAB*, *soxRS*, *emrAB* and *acrAB* (reviewed in [26]). Operons are multigenic units containing a regulator gene, an operator-promoter region and one or more other genes whose expression is regulated by the product of the regulator gene. Each operon’s regulator gene encodes a repressor protein which senses the presence of the antibiotic. There is a great deal of homology among the genes comprising different operons. For example, the proteins encoded by the regulator genes, *marR* and *emrR*, are two of the 15 proteins belonging to the MarR family, members of which are found, not only in Gram-negative bacteria inhabiting the gut, but also in Gram-positive bacteria, and the mycobacteria, including plant and animal pathogens, as well as free-living species. (The convention in genetics is to italicize the genes and to represent the corresponding protein encoded in non-italicized form.)

One member of the MarR family, HpcR, found in soil-dwelling *E.*

coli C strains, is a repressor of an operon encoding the enzymes that break down homoprotocatechuate (Hpc), a phenolic compound derived from plants. The repressor protein, HpcR, binds to the operator and represses the synthesis of enzymes that break down Hpc when Hpc is *absent*. But when Hpc is present, the repressor protein binds to it instead, freeing the operator, so that transcription and synthesis of the enzymes can take place. These observations suggest that naturally occurring noxious substances in the environment are the original inducers of the multiple antibiotic resistance operons.

Further observations in support of the idea are that other naturally occurring compounds, such as salicylic acid, dinitrophenol and naphthoquinones, stimulate the expression of both *soxRS* and *marRAB*. The natural naphthoquinolones, plumagin and menadine, are effective inducers at relatively low concentrations, and mutants lacking both the *marRAB* and *soxRS* operons are notably hypersensitive to those compounds, while sensitivity to salicylic acid is only marginally affected. The *marRAB* operon expression is enhanced at 30°C compared with 37°C, as consistent with its origin in free-living bacteria [26]. The multiple antibiotic resistance operons have, therefore, evolved from free-living bacteria. The enteric bacteria have simply reactivated or mutated the genes in the operons to allow them to cope with drugs and antibiotics. This class of mechanisms is reminiscent of the multidrug resistance which all mammalian and plant cells are able to develop in the presence of sublethal doses of noxious substances (see the next chapter).

Chapter 4

“Random” versus “Directed” Mutations

To measure the rate of “spontaneous” mutations to antibiotic resistance in a bacterial strain, a small amount of a dilute suspension of bacteria is spread on a nutrient agar-plate impregnated with a concentration of the antibiotic high enough to kill nonresistant bacteria, so that only those with a *pre-existing* mutation which happens to confer resistance will survive and form colonies. The spontaneous mutation rate for antibiotic resistance estimated in this way is typically 10^{-6} or lower, and is about the same for all other genes in the genome. This is the rate of *random* mutations, in other words, mutations that have no direct correlation to the environment, or the “selective” regime. Based on these estimates, microbiologists did not believe that resistance to antibiotics would pose a problem when antibiotics first came into use [24]. So why did antibiotic resistance evolve and spread so rapidly?

The usual explanation for antibiotic resistance, according to neo-Darwinian theory, is that it is due to the intense “selection pressures” exerted by the antibiotics, so that even extremely low rates of spontaneous random mutations that happen to confer resistance to the antibiotic will be rapidly selected for, while those which do not happen to have the mutation will die out (see, for example, [32]). Antibiotic resistance has been assumed to be a classic case of the natural selection of random mutations. What actually happens is something else.

First of all, the mutations are not random in the sense that they

pre-exist in the population, before exposure to antibiotics. All bacteria, with the exception of those that live in hot springs, are killed by boiling or cooking, which is therefore the surest way to prevent infection. They can also be killed by high-enough concentrations of antiseptics or antibiotics to which they are susceptible. However, at lower concentrations of the noxious agent, the bacterium may fail to grow at first, but after some time, may develop the required resistance to the drug which enables it to grow, multiply and form colonies. This ability to develop drug resistance is common to cells of all species — bacteria, fungi, plants and animals without exception [51-54]. It is a physiological response shared by *all* the cells or organisms of the population, and is not due to selection of pre-existing random mutations. It is similar to the “directed mutations” or “adaptive mutations” (see Chapter 5 below), whereby starving, non-growing bacteria eventually acquire mutations that enable them to feed on new substrates [55-57].

Secondly, competition is irrelevant among bacteria in the evolution of antibiotic resistance: they share their most valuable assets for survival — genes encoding resistance mechanisms against antibiotics — by passing them on horizontally between species.

In fact, the evolution of antibiotic resistance has turned out to be a paradigm case of the “fluid genome” in the new genetics, in which genes and genomes can mutate and undergo other changes when the ecological conditions call for them, and in which genes can even travel through the external environment from one organism to another [35, 58]. We shall first consider “adaptive mutations” in the broader context of the lessons which have been learned from molecular genetics within the past two decades.

Chapter 5

“Natural” Genetic Engineering and “Adaptive Mutations”

In a recent review, microbial geneticist Shapiro [59] offers an appropriate perspective of bacterial evolution that sums up the recent discoveries of molecular genetics on how genomes are organized, on the biochemical mechanisms of genetic change and the cellular control networks regulating genetic change.

Bacterial genomes are organized in modular fashion at successive levels of a genetic hierarchy. At the lowest level of the hierarchy are genes composed of different domains (or subunits) that are re-shuffled and recombined to give different genes. Different combinations of similar genes give rise to multigene units or operons, and different combinations of operons make up larger units or “regulons” for complex functions such as chemotaxis (movements directed by specific chemicals), symbiosis and protein secretion. Regulons may coincide with independently replicating “replicons”, such as plasmids and viruses, each with its own signal for replication (called origin of replication). Thus, mosaicism, or cut-and-splice genetic recombination, is involved in every level of genome organization, and plays a key role in generating genetic diversity rapidly. But these mechanisms are by no means random.

Bacteria may have “little tolerance for purely random variability” [59]. Bacterial cells possess a wide range of repair and proof-reading functions to remove accidental changes to DNA sequences and correct errors resulting from physiological insults. But the same cells also possess numerous biochemical mechanisms for chang-

ing and reorganizing DNA, suggesting that such changes are the result of the “natural genetic engineering” ability of bacteria to respond to environmental challenges.

The possibility of “natural” genetic engineering mechanisms responding to the environment gained considerable support in the discovery of “adaptive mutation”, in which starving bacteria acquire the appropriate genetic mutation enabling them to metabolize a new substrate, when the substrate is present in the “selective” medium. A wide variety of mechanisms are involved in generating adaptive mutations, including point mutations (single base changes), deletions or insertions of nucleotides, conversions of gene sequences from one to another, site-specific integration of genetic material, DNA rearrangement and transposition (gene jumping) (reviewed in [59]).

There is much current debate as to whether those mutations are specifically “directed” by the environmental challenge, or simply the consequence of a hyper-mutable state of stressed cells that generates many mutations indiscriminately at a high rate, increasing the chances of a lucky hit. In either case, however, it means that genes and genomes change as the result of built-in mechanisms that are subject to metabolic and environmental feedback regulation [57]. As Shapiro points out, the ability to activate those mechanisms under stress can significantly accelerate evolutionary change in crisis without threatening genetic stability under ordinary circumstances. He surmises that further studies of mobile genetic elements and DNA rearrangements will ultimately uncover truly directed mutations. Just as signal-transduction systems of the cell can direct the transcriptional apparatus to specific locations in the genome to activate those genes, so it is possible that the mutational apparatus(es) can be similarly directed to mutate specific genes.

Shapiro regards the evolution of antibiotic resistance and virulence as clear examples of “natural” genetic engineering mechanisms at work, and goes no further than that. This can easily lead human

genetic engineers to justify what they do on grounds that bacteria themselves have been at it, probably for hundreds of millions of years. That kind of reasoning is fallacious and dangerous. As recent studies indicate, “normal circumstances” for the vast majority of microbes in the environment as well as in our bodies may be a non-proliferating but metabolically active, non-virulent state [60, 61]. This state is associated with layers of extracellular matrices or “biofilms” in which typically mixed communities of microbes are embedded, where they enjoy a cooperative, multicellular existence rather like the cells and tissues in our body (see Ho [35, Chapter 14]).

Furthermore, there is nothing “natural” about the “man-made” environmental conditions that have provoked microbes to engineer their genes and genomes. The discharge of toxic wastes, the destruction of the environment, and the overuse and abuse of antibiotics have already thrown the “natural” ecology of microbes seriously out of balance. And over the past 20 years, the extensive manipulation of genes and genomes by human genetic engineers is very likely to have exacerbated the situation. The ecology of microbes consists, not only of the usually understood interrelationships with other organisms, but also of genes within the genome, of the genome within the physiological state of the cell and the organism, which is in constant intercommunication with its environment. Disease, we are told, cannot be understood apart from the ecological context [62]. More importantly, it must be understood in terms of the nested levels in the ecology of genes [36] and microbes, which is inextricably entangled with the ecology of human beings.

Chapter 6

Horizontal Gene Transfer

The major role of horizontal gene transfer in spreading antibiotic resistance has only been recognized since the late 1980s, when a Conference on Antibiotic Resistance was co-sponsored by the Environment Protection Agency and the National Science Foundation of the USA to look into the mechanisms and factors affecting such gene transfers [63]. The first definitive evidence for horizontal gene transfer came from DNA sequence analysis of the genes for neomycin-kanamycin resistance from *Staphylococcus aureus*, *Streptococcus* and *Campylobacter* sp. [64] which were found to be essentially identical. Identity of sequence not only gives evidence for horizontal gene transfer, but also indicates that the transfer is a very recent event. Further evidence of horizontal gene transfer was subsequently obtained for a wide range of antibiotic resistances, by DNA sequencing or other methods of characterizing DNA [65-70].

6.1 The scope of horizontal gene transfer

The scope of horizontal gene transfer is essentially the entire biosphere [13, 14, 36, 38, 71-77]. Genes are found to have been transferred, not only among microorganisms and viruses, but among eukaryotic species and across traditional Kingdoms of organisms. Among eukaryotes, the main transfers have involved transposable elements [36, 77] such as the *P* element among *Drosophila*ids, and transposable introns (non-coding segments of genes) among lower eukaryotes and bacteria [36]. Some especially promiscuous ele-

ments such as *mariner* have been found in arthropods and vertebrates, including primates and humans, where it is associated with a neurological wasting disease [78]. Gene transfers mediated by transposable elements do not require DNA base sequence homologies.

Transfers have occurred from bacteria to higher plants and *vice versa*. The best known example is the direct demonstration of transfer between the soil bacterium, *Agrobacterium* and plants. In a process bearing a strong resemblance to conjugation between bacteria (see section 7.3), the tumour (*T*) segment of the tumour-inducing (*Ti*) bacterial plasmid is transferred and incorporated into the plant genome [79, 80]. However, it must be noted that nearly *all* cases of directly demonstrated horizontal gene transfers, especially those involving phylogenetically distant species, made use of already modified hybrid shuttle vectors that can transfer between species and replicate in both. These shuttle vectors possess signals for replication (origins of replication) in more than one species as well as the signal for DNA transfer (origin of transfer), and are hence much more likely to be successful in horizontal transfer than unmodified plasmids found naturally. The *Ti* plasmid is, indeed, the basis of a gene transfer vector system widely used for genetically engineering crop-plants (see Appendix II).

Cross-Kingdom horizontal gene transfer by conjugation has also been demonstrated between bacteria and yeast using shuttle vectors derived from broad host-range promiscuous plasmids (see section 7.3) which are already transferable between many bacterial species [71-74, 81]. Such vectors can even substitute for the *Ti* plasmid in transferring genes from *Agrobacterium* to plants [82]. Recent evidence documented the direct transfer of transgenes and marker genes from transgenic plants to soil fungi [83] and soil bacteria [84], indicating that secondary, unintended gene transfers can occur from genetically engineered crop-plants which are now released commercially into the environment. Despite the title of their publication, Schluter *et al.* [84] actually observed a high "optimal"

gene transfer frequency of 6.2×10^{-3} in the laboratory, from which they “calculated” a frequency of 2.0×10^{-17} under extrapolated “natural conditions”. The natural conditions, are of course, largely unknown [36]. In a recent Workshop at the German Environmental Protection Agency, two research groups reported secondary transfer of antibiotic resistance marker genes from transgenic plants to soil microorganisms [85, 86]. Debris and exudates from transgenic plants will, therefore, be expected to transform bacteria and other microorganisms in the soil. *E. coli* cells have been successfully transformed in the laboratory by T-DNA, simply using total DNA from transgenic plant; and the T-DNA vector was recovered as a circular plasmid in the transformed cells [79]. In addition, other vectors such as viruses, insects and nematodes can also mediate unintended, secondary horizontal gene transfer [36].

Mammalian cells are known to take up foreign genes by a number of mechanisms including anastomosis (fusion), endosymbiosis (accommodating microorganisms and viruses as symbionts), phagocytosis (engulfing viruses and other particles) [87, 88] and also endocytosis, a process dependent on specific cell-membrane receptors binding to DNA or to macromolecules (most frequently, proteins) complexed to DNA, so that the DNA can be internalized [36]. It has even been suspected that gene transfer from bacteria to animal cells can occur by conjugation, a mating process previously thought to be confined to bacterial cells (see section 11.3). The ability of certain viruses (retroviruses) to capture and transfer genes among mammalian cells is well known and is associated with many animal cancers (see entries in [89]). Numerous illegitimate recombinations requiring no sequence homologies are due to retroviral insertion into the genome transferring genes horizontally between individuals of the same or different species [36]. In short, a given gene from any organism has the potential to spread to all other organisms in the biosphere [13, 14].

However, DNA uptake by itself is not sufficient for a successful gene transfer. Important barriers operate *after* the uptake of for-

eign DNA which break down, or otherwise block the integration, use and maintenance of the foreign genes [40, 77]. Plasmid DNA requires an origin of replication that is recognized and used by the host cell before it can be replicated and maintained. Similarly, promoter sequences which can be recognized by the host are necessary for expressing the viral genes that make the virus, as well as expressing any other foreign genes carried by the virus. (Though, of course, origin of replication and promoter sequences can be acquired by recombination.) However, degraded DNA coding for parts or domains of proteins — or short regulatory motifs that bind transcription factors or otherwise enhance or repress gene expression — may also be integrated, with significant genetic effects including cancer [36]. Recombination of domains is already known to be a major factor in generating new proteins [59].

While the host-cell DNA has its own origins of replication, methylation patterns (chemical markers) and promoter sequences which are specific to the species, artificial gene transfer vectors and other manipulated DNA have recombined origins of replication and promoter sequences from different species which can be recognized by different host species. In addition, they are designed to overcome the restriction systems which break down and “silence” foreign DNA. *Thus, genetic engineering may have created new avenues and possibilities for increasing the scope of horizontal gene transfer.* We shall consider this in more detail in Chapter 10.

The scope of horizontal gene transfer among bacterial species has to be appreciated in the light of the revised phylogeny of the living world based on comparing ribosomal RNA (rRNA) sequences, a procedure first proposed by Carl Woese [90]. Ribosomal RNAs are the most conservative molecules in evolution, and have not been subject to horizontal transfer between species. For these reasons, phylogenies based on rRNA sequences are generally considered to be the most reliable.

Comparisons of rRNA and other conserved sequences give a uni-

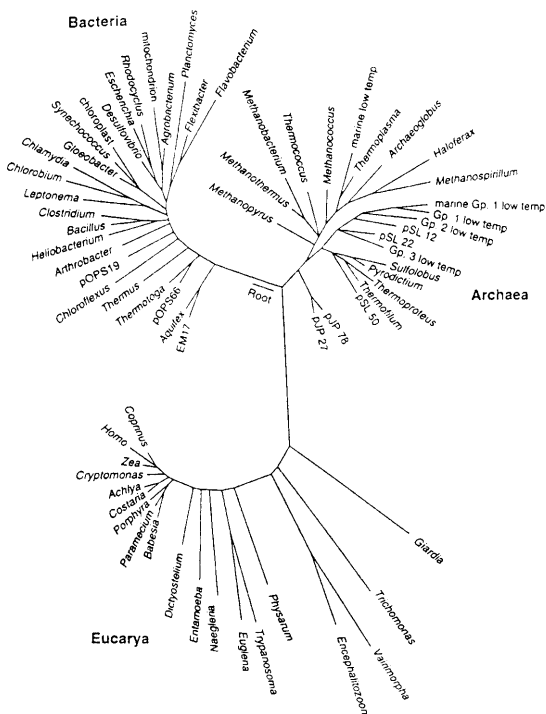


Figure 3. The universal phylogenetic tree of the living world.

versal phylogenetic tree (Figure 3) [91], revealing, for the first time, the true phylogenetic status of bacterial species. The tree is tripartite, with Bacteria (Eubacteria), Archaea (Archebacteria) and Eucarya (Eukaryotes) forming three more or less equal Domains. The traditional Kingdoms — Plants, Animals and Fungi (*Coprinus*) — are but twigs on a small peripheral branch of the Eucarya Domain, each twig having the same phylogenetic status as traditional Orders of bacteria. Within the Bacteria Domain, *E. coli* (a Gram-negative bacterium) is as distant from the Gram-positive bacteria, *Bacillus*, or *Heliobacterium*, as *Homo sapiens* is from the single-celled ciliate, *Paramecium*.

But even the revised phylogeny of the living world greatly underestimates the genetic diversity of Bacteria. The known bacterial species are less than 1% of all species that exist, as more than 99% cannot be cultivated in the laboratory by routine techniques [91]. This highlights the dangers of releasing genetically engineered microorganisms or DNA into the environment.

All known pathogens belong to the Bacteria and some to the Eucarya Domain. None belongs to Archaea despite the fact that some archaean species share our environment and are part of our normal bowel flora. Strauss and Falkow [92] suggest there may be more fundamental differences between organisms that can become pathogens and those that do not. Another reason, however, may be that, until fairly recently, archaean organisms have not been genetically manipulated to the same extent as have bacterial species. Will the current trend towards increasing manipulation of archaean species also turn previously benign organisms into pathogens? Judging from the recent history of genetic engineering, it may only take a few years of intensive genetic manipulation of new (archaean) species for new pathogens to emerge. There are essentially no barriers left to horizontal gene transfer and recombination, particularly as the result of genetic engineering.

Chapter 7

Mechanisms of Horizontal Gene Transfer

There are three main mechanisms for gene transfer in bacteria: a) by direct uptake of DNA (transformation) b) through genes being carried by viruses that infect bacterial cells (transduction) and c) through a mating process requiring cell-to-cell contact (conjugation). These processes have been studied most extensively in *E. coli*, although it is becoming clear that what is true for *E. coli* does not necessarily apply to other species. The scope of our present ignorance is enormous, but what is known already gives us serious cause for concern.

7.1 Transformation frequencies are high

Transformation among microbes in the environment has been extensively reviewed recently [36-38], showing that it is extremely widespread. The process involves DNA being released into the environment, its binding to the surface of bacteria, entry into the cell and recombination with the bacterial chromosome, or in the case of plasmids, reconstituting the plasmid. All these stages are facilitated by bacterial proteins. Both chromosomal and plasmid DNA are able to transform bacteria, the frequency of transformation varying with the physiological state of the cell, diffusible factors excreted from the bacteria and with the presence of salts in the environment. In addition, the nature of the DNA, its source, size, and state may also be important. Generally, transformation is most frequent if the DNA is from the same species, as its successful integration into the recipient genome depends on the degree of ho-

mology, i.e., the similarity in base sequence between the transforming DNA and the host DNA. It also depends on the DNA being resistant to the restriction enzymes in the host cell which break down foreign DNA. In one experiment, brief heat treatment (9 minutes at 48.5°C) dramatically increased the competence of cells for receiving foreign DNA by four orders of magnitude [73]. This was attributed to the heat inactivation of restriction enzymes, thereby increasing the success of gene transfer. Cross-species, cross-genera and even cross-order transfers have been observed with chromosomal DNA. Plasmid DNA, in particular, has effected cross-Kingdom transformations among Eubacteria, Proteobacteria and Cyanobacteria [37].

DNA is not only released into the environment when the cells die, but is actively excreted by living cells during growth. Some species export DNA wrapped in membrane-bound vesicles. The DNA in a culture slime can be more than 40% of the dry weight. Thus, the environment is extremely rich in DNA. Marine water contains between 0.2 to 44 microgram (μg) per litre, and fresh water contains between 0.5 to 7.8 μg per litre; while freshwater sediment has an upper concentration of 1 μg per gram. Although enzymes breaking down DNA (deoxyribonucleases or DNases) are found in the environment, DNA is protected from degradation by adsorbing to detritus, humic acid, and in particular, clay and sand particles. Adsorbed DNA is equally efficient in transforming cells. Thus, the half-life of DNA (time for half of the DNA to be broken down) in soil is 9.1 hours (h) for loamy sandy soil, 15.1 h for silty clay soil and 28.2 h for clay soil. While half-lives in waste water are typically fractions of an hour, those in fresh water and marine water are 3 to 5 h with high values of 45 to 83 h on the ocean surface, and extremely high values of 140 and 235 hours for marine sediment [37]. Adsorption of DNA to solid particles is a very rapid process, which means that DNA released into the environment can survive indefinitely and maintain its potential to transform other organisms.

Transformation may be a major route of horizontal gene transfers: frequencies obtained under different environmental conditions, *using artificial vectors and markers* are found to be generally quite high, many ranging between 10^{-2} to 10^{-5} transformant per viable cell. In fact, transformation frequencies are often *higher* under natural conditions than in the laboratory. In a recent study in soil microcosms, activity due to earthworms was found to significantly enhance gene transfer between spatially separated bacterial species inoculated into the soil [93]. A special form of bi-directional transformation by cell contact and fusion is now known to be widespread [38].

The release, or escape, of naked recombinant DNA into the environment is a growing ecological problem, not only in the form of transgenic organisms, but also in vaccines, gene therapy vectors, antisense oligonucleotides (short sequences of RNA complementary to the base sequence in the transcript of the genes) and ribozymes (RNA that act as enzymes to splice out intron sequences from transcribed genes) — used in various forms of gene therapy [36]. As mentioned earlier, even short sequences can have significant genetic effects when integrated into the genome of organisms for which they are not intended.

The hazard of naked DNA is highlighted by the discovery that some viral DNA may infect non-permissive hosts for which the virus itself is non-infective [36]. Intravenous injections of naked, circular polyoma viral DNA into rabbits and mice gave a powerful genetic expression with the development of antibodies and the production of new virus particles [94], whereas the native virus had no effect. Similarly, intravenous injections of recombinant plasmids in liposomes (vesicles made from membrane lipids) gave gene expression in several organs including the ovaries, indicating the possibility of an inherited genetic change [36]. These findings are the basis for developing many recombinant DNA vaccines [95], for which ecological risk assessment has hardly even been considered.

DNA vaccines are inherently dangerous due to their ability to infect non-permissive hosts, thus crossing wide species barriers, with many opportunities for recombination to generate new viruses. Recombinant DNA vaccines are worse, as even small modifications of a virus can result in significant, unpredictable changes in its host range and virulence [36]. In addition, they are more prone to recombine with other viruses to generate new viruses. Similar hazards are associated with the gene therapy vectors which are currently being developed (see also section 11.3).

7.2 Transduction is substantial in aquatic environments

Transduction takes place by means of bacteriophages which are usually host specific, and are hence not expected to transfer genes between unrelated species. However, promiscuous phages with broad host ranges are capable of transducing genes between unrelated species [38]. A bacteriophage consists of viral DNA wrapped in a protein coat, which binds to specific receptor sites on the bacteria. Once bound to the receptor sites, the bacteriophage injects its DNA into the bacterium. The internalized phage DNA either directs the synthesis of many bacteriophages which eventually break open (lyse) the cell, or the phage DNA may insert itself into the bacterial chromosome (as provirus or prophage) to become replicated with the chromosome. The bacteriophage can transfer genes by mispackaging a piece of bacterial DNA in the viral protein coat, or by packaging a viral DNA that has a piece of bacterial DNA incorporated into it. When such a virus infects a second cell, the bacterial DNA is transferred and may then become integrated into the new bacterial chromosome. The transducing phage may serve as a vehicle for a transposon that can then establish itself in an unrelated host, even though the phage DNA cannot do so, and hence contribute to cross-species, cross-genera gene transfers.

Also, bacteriophages themselves evolve by horizontal gene transfer and recombination, leading to broadening of their host ranges [96]. One study [97] shows that there may be as many as 10^6 bacterio-

phages per millilitre in aquatic environments, so that one third of the total bacterial population is subjected to a phage attack every 24 hours, each capable of transducing foreign genes into the cell. Recently, Miller [98] reports 10^{11} virus particles per millilitre in fresh and marine waters.

Phages are now known to be intimately involved in the horizontal transfer of entire blocks of virulence genes into the bacterial chromosome, turning a previously benign strain into a pathogen in a single step; and co-integrates of phage and plasmids are also involved in trafficking blocks of virulence genes (see section 9.1).

7.3 Conjugation is essentially promiscuous

Conjugation was previously thought to be a species-specific process requiring complex, complementary interactions between the genetic "donor" and the genetic "recipient". Conjugation depends on certain conjugative plasmids, extrachromosomal pieces of DNA that are replicated independently of the chromosome. They carry genes required for the process of conjugation. These plasmids fall into different incompatibility groups. Ippen-Ihler and Skurray [99] listed seven or more incompatibility groups of over a hundred known plasmids, *most of them carrying one or more antibiotic resistance genes*. Plasmids in the same incompatibility group cannot be maintained in the same bacterial cell at the same time. However, there are common mechanisms for gene transfer that are interchangeable. One of these codes for the pilus, a tube-like structure connecting the donor with recipient cells in conjugation, through which plasmid DNA passes from the donor to the recipient. In other cases, such as the genes involved in the transfer of the plasmid, the gene products are specific to the plasmid and its host, and not interchangeable. Several of the incompatibility groups comprise "promiscuous plasmids" with broad host ranges.

Conjugative plasmids can integrate themselves into the bacterial chromosome. In such cases, when conjugation takes place, the in-

tegrated plasmid will drag the bacterial chromosome with it across the conjugation tube from donor to recipient, leading to transfer of bacterial DNA as well as plasmid DNA. Donor DNA will then recombine with recipient DNA to generate new genetic recombinations. The DNA is thought to be passed in a single-stranded form, which is resistant to breakdown by enzymes attacking double-stranded DNA (nucleases and restriction enzymes), thus ensuring a high success rate of gene transfer. The single-stranded DNA then directs the synthesis of the complementary strand to restore double-stranded DNA in both donor and recipient.

Recent studies indicate that conjugation has an extraordinarily wide host range, involving diverse, complex mechanisms that are not yet well understood [38, 100]. "Promiscuous" plasmids overcome species barriers, transferring genes promiscuously between phylogenetically distant species. At least several of the promiscuous conjugative plasmids have been exploited in constructing artificial shuttle vectors between *E. coli* and other distant species. One promiscuous conjugative plasmid is routinely used in *Agrobacterium*-mediated gene transfer to make transgenic plants (see Table A.2 of Appendix II). Shuttle vectors are essentially unstoppable, as they typically carry the origins of replication for both species as well as the origin of conjugative transfer. The strength of conjugative transfer is in the single-stranded DNA transferred directly, which escapes destruction by restriction enzymes. While the shuttle vectors are constructed to require "helper" transfer functions provided by other plasmids, those plasmids can be present in the donor or recipient, or in a third species altogether. These "tripartite" matings can easily occur in all environments, which are full of known and especially, unknown bacterial species.

In addition to conjugative plasmids, conjugative transposons mediate their own transfer to recipient cells during conjugation and insert into the recipient chromosome [101, 102]. They can also jump

from one site to another on the same chromosome, or from the chromosome into plasmids and *vice versa*. These have also been exploited in constructing gene transfer vectors (see Table A.2 of Appendix II). A special class of such conjugative transposons, "integrons" [103-105], is thought to be especially involved in the evolution of the multi-resistance R plasmids within the past five decades [24]. An integron carries its own gene encoding an enzyme, integrase, that catalyzes the site-specific integration of antibiotic resistance gene "cassettes" within the integron, such that each inserted "cassette" is provided with a ready-made promoter for expression. An integron can carry several cassettes, each encoding a different antibiotic resistance. The integron can facilitate recombination between cassettes to form exotic gene-fusions that code for multifunctional proteins. Integrons can also jump from the bacterial chromosome into plasmids and are transferred to another bacterium during conjugation.

During conjugation, genetic material not only goes from donor to recipient, but also in reverse, from recipient to donor, in a recently discovered process, retrotransfer [38].

7.4 Effects of environmental pollutants on horizontal gene transfer

Horizontal gene transfer is sensitive to many physical and chemical factors. In routine laboratory procedure for genetic transformation, heavy metals, singly or in combination, are used to greatly increase the competence of cells for transformation, as are enzymes such as muraminidases or proteases. Freezing and thawing, treatment with EDTA or other chelating agents, exposing cells to electric fields, or to DNA wrapped in liposomes, are all effective in increasing the frequency of transformation. Yet, very few experimental investigations have been carried out into the effects of environmental pollutants on horizontal gene transfer. This is a glaring omission in view of the hundreds of xenobiotics that currently circulate in our environment, to which genetic engineering

biotechnology is releasing or discharging large volumes of agents — transgenic plants and animals, microorganisms, vectors, vaccines, and a range of naked DNA and oligonucleotides — all capable of taking part in horizontal gene transfers [36]. We shall define xenobiotics as compounds released by human beings into the environment in concentrations that create harmful effects.

Xenobiotics can affect horizontal gene transfer in two ways [36]. Some xenobiotics mutate DNA, thus influencing its uptake into cells. Kipling and Kearsey [106] have reported examples of minor changes in a DNA sequence which alter the host range of a transferable genetic element. Other xenobiotics can affect the cell membrane and/or intracellular functions thereby influencing the ability of cells to take up DNA, or to engage in conjugation and other forms of gene exchange.

As mentioned in section 1.3, antibiotics can increase horizontal gene transfers 10- to 10,000-fold. Antibiotics are heavily used in medicine and intensive agriculture, especially in fish farming, and constitute an important class of xenobiotics that already affect horizontal gene transfer significantly. It is known that ancestral antibiotic resistance genes originated from antibiotic-producing microorganisms themselves. The possibility has been suggested [24], therefore, that what we call antibiotics may actually be sex hormones for the bacteria, enhancing conjugation between cells. The process is naturally regulated by the product of the “antibiotic resistance” gene, which shuts off the signal. Thus, the use of antibiotics not only leads to directed mutations of antibiotic resistance genes (see Chapter 5), it promotes the spread of those very genes among pathogens. The case for phasing out the use of antibiotics in intensive agriculture and fish farming is overwhelming. Antibiotics are also routinely used in genetic engineering biotechnology to select for transformed cells and recombinants.

The importance of synergistic interactions between xenobiotics is increasingly recognized. Recent research has shown that chemi-

cals that are apparently harmless when tested singly, can have enhanced effects in mixtures at very low concentrations [107]. As xenobiotics never occur singly in the environment, it is necessary to test drugs and chemicals in combination for a realistic risk assessment. But this is a logistically and financially impossible task. To test just 1,000 of the commonest toxic chemicals in combinations of three (at a standardized dosage) would require at least 166 million different experiments. An increasing number of scientists are calling for a drastic reduction in the number of chemicals to which we are exposed: they wish to see the introduction of reverse onus, in which a chemical must be regarded as potentially harmful until proven otherwise. Xenobiotics are not made by living organisms, possibly for the good reason that they are incompatible with healthy cellular activities. The synergistic effects of xenobiotics on horizontal gene transfer are, for the most part, unknown and unpredictable.

The precautionary principle demands that we should strive to limit the release/escape of genetically engineered organisms, naked DNA and RNA sequences into the environment as much as possible.

Chapter 8

Horizontal Transfer of Antibiotic Resistance Genes

There could be very few barriers remaining to horizontal gene transfer. Antibiotic resistance genes, especially those carried on R (Resistance) plasmids and transposons can, in principle, cross species as well as genera and Kingdoms and even Domains. Evidence for transfer is obtained wherever and whenever it has been looked for, either by sequence analyses or by direct experimental investigations, as mentioned in Chapters 1 and 6 . We cite further examples below.

Tetracycline resistance genes are now found to be shared between many genera of bacteria. One of them, the *TetL* gene, is found in *Actinomyces*, *Clostridium*, *Enterococcus*, *Listeria*, *Peptostreptococcus*, *Streptococcus*, *Staphylococcus* and *Bacillus*. Another gene, the *TetK*, is found in *Bacillus*, *Clostridium*, *Enterococcus*, *Eubacterium*, *Listeria*, *Peptostreptococcus*, *Staphylococcus* and *Streptococcus* [108-110]. More recently, both *TetL* and *TetK* have been found for the first time in soil bacterial species belonging to the genera *Mycobacterium* and *Streptomyces*, which can cause soft-tissue and skin infections [111]. Antibiotic-producing *Streptomyces* is believed to be the ancestral source of many aminoglycoside antibiotic resistance genes, but it is now gaining more of these genes back from the other species. These findings are of particular significance, as they “suggest the potential for the spread of an antibiotic resistance gene into all environmental mycobacteria, including *Mycobacterium leprae*” [111, p. 1411].

Even chromosomally encoded penicillin resistance genes (penicillin-binding proteins, pbps, required for the uptake of penicillin) are readily transferred. Transfers of *pbp* genes were found between *Streptococcus pneumoniae* and *Neisseria gonorrhoeae* which subsequently recombined to generate new hybrid genes [112].

Chapter 9

Horizontal Gene Transfer and the Emergence of New and Old Virulent Strains

The very same genetic mechanisms for horizontal gene transfer have been involved in the emergence of virulence among old and new pathogens since the mid-980s. For example, there has been an increase, since then, in sporadic cases of very severe invasive *Streptococcus pyogenes* infections in Europe, North America and elsewhere. In an analysis of 108 isolates from patients in the USA with streptococcal toxic shock syndrome [113], a toxin frequently found is encoded by a gene, *SpeA*, belonging to a bacteriophage, which has become inserted into the bacterial genome. This gene has spread horizontally among different strains of *S. pyogenes*.

In addition, genes encoding membrane surface proteins — which determine binding properties and virulence of the bacteria — have also undergone numerous recombination events subsequent to horizontal gene transfer, giving rise to mosaic structures of the genes. This is superimposed on the accumulation of point mutations acquired as the result of interactions of the pathogens with the immunological system of the human host, which make antibodies against the membrane surface proteins of the bacteria. A similar picture emerged on analysis of group A streptococci isolated from a cluster of cases of serious infections over a three-month period in Tayside, Scotland, in 1993 [21]. DNA sequence analysis of virulence factors suggests that they may have been recently acquired by horizontal gene transfer.

In the past, only *Vibrio cholerae* strains of the type O1 are known to have caused epidemics, while non-O1 strains are associated with sporadic cases. However, the recent epidemic in Asia was caused by a non-O1 strain, *Vibrio cholerae* O139. It turns out that the new strain is identical to an earlier pandemic strain, O1 El Tor, except for proteins involved in the capsule and the O-antigen synthesis (which is used in typing the strains). This was due to the acquisition of DNA inserted into, and replacing part of, the O-antigen gene cluster. This suggests that the O139 strain arose by horizontal gene transfer from a non-O1 strain into a type-O1 strain [15-17].

Pathogenic mycoplasmas possess proteins called adhesins and related accessory proteins which are required for adhering to cells and subsequent disease development. *Mycoplasma genitalium*, implicated in urethritis, pneumonia, arthritis and AIDS progression, was found to encode one adhesin and two adhesin-related proteins that shared substantial sequence similarities, as well as genome organization, with those of another species, *M. pneumoniae* [114]. These were attributed to horizontal gene transfer events between the two species.

Most disturbing of all is the recent discovery that at least 10 unrelated bacterial pathogens, causing diseases from bubonic plague to tree blight, share an entire set of genes for invading host cells, which have spread by horizontal gene transfer, as they reside as a block, often in the bacterial chromosome, forming "pathogenicity islands" (see section 9.1) [23]. More than 20 genes are involved in a system that secretes damaging proteins directly into host cells. They were first discovered in *Yersinia*, a genus of several species that cause human disease including bubonic plague and intestinal infections. At least some of the proteins are typical of eukaryotic cells, and are targeted at eukaryotic biochemistry [115], leading to the speculation that the genes were originally acquired from eukaryotic cells. We shall look at some of these systems in more detail later.

Like antibiotic resistance genes, many virulence genes reside on transposons or plasmids. For example, the ST-enterotoxin genes of *E. coli* are part of a transposon; while important virulence factors of Gram-negative pathogens (*Shigella flexneri*, *Salmonella* spp., *Yersinia* spp.) as well as Gram-positive pathogens (*Clostridium tetani*) are encoded in plasmids. Other factors, including the Shiga-like toxins of *E. coli*, the cholera toxin of *Vibrio cholerae*, diphtheria toxin of *Corynebacterium diphtheriae*, neurotoxins of *Clostridium botulinum* and cytotoxin of *Pseudomonas aeruginosa*, are encoded by bacteriophages [116, 117]. All these toxins and virulence factors obviously have the capacity to spread horizontally and to create new pathogens by recombination.

Virulence factors are often also located on the chromosome, where they form large blocks, which came to be called “pathogenicity islands.”

9.1 Pathogenicity islands

The term “pathogenicity island” (*Pai*) refers to large chromosomal regions in pathogenic bacteria that encode virulence genes acquired by bacteria from unrelated organisms. Hacker *et al.* [118] reviewed the subject recently. They define a *Pai* according to the following criteria:

- a. Carriage of (often many) virulence genes.
- b. Presence in pathogenic strains, and absence or sporadic distribution in less pathogenic strains of one species or a related species.
- c. Different G+C content in comparison to DNA of host bacteria. (The G+C content, or content of the bases, guanine and cytosine, is characteristic of DNA from each species; and difference from host DNA is indicative of a foreign source.)

- d. Occupation of large chromosomal regions (often more than 30 kilobases or kb).
- e. Representing compact, distinct genetic units, often flanked by direct repeats (like many viruses and transposons).
- f. Association with transfer RNA (tRNA) genes and/or insertion sequence (*IS*), a special class of mobile genetic elements, at their boundaries.
- g. Presence of (often cryptic) mobility genes (*IS* elements, integrases, transposases, origins of plasmid replication).
- h. Instability.

A bacterial pathogen may have one or more pathogenicity islands. For example, uropathogenic (causing infection of the urinary tract) *E. coli* strain 536 has two *Pais*: *Pai I* encodes only α -haemolysin, responsible for haemolysis, while *Pai II* carries the α -haemolysin gene (*hly*), as well as the P-related fimbriae gene (*prf*) involved in attachment of the bacterium to the host cell. *Pai I* is flanked by the *selC* gene encoding the selenocysteine-specific tRNA and two direct 16 base-pair (bp) repeats, while *Pai II* is associated with *leuX*, which encodes a leucyl tRNA, and two direct 18 bp repeats. The direct repeats in both cases are attachment sites for bacteriophages (ϕ R73 in *Pai I*, and P4 in *Pai II*). Cryptic integrase genes (for integration into the bacterial genome) of these phages are located near the respective tRNA genes. Thus, the *Pais* probably originate from integrated prophages which have picked up a range of virulence genes to turn them into pathogenicity islands.

In addition, sequences homologous to the origins of replication of plasmids, parts of *IS* elements, and “recombination hot spots” (sites which are most frequently involved in recombination) are also present. All these indicate that *Pais* are highly mosaic, and many

recombination events have contributed to their evolution. Have some of these recombinations been inadvertently created by genetic engineering?

Phages, plasmids and pathogenicity islands are involved in rapid evolutionary changes of bacteria by “quantum leaps”. *Pais* often exhibit features of both plasmids and phages, reflecting the formation of co-integrates of both types of extra-chromosomal elements. The acquisition of new genes following horizontal gene transfer results in the generation of new variants of pathogens. Drug-resistant strains of *Mycobacterium tuberculosis* and *Streptococcus pneumoniae*, the new *V. cholerae* serotype 0139, *E. coli* 0157, *Haemophilus influenzae* biotype *aegyptius*, are examples of such new types of pathogens generated by horizontal gene transfer.

Enteropathogenic bacterial strains (infecting the gastrointestinal tract), including *E. coli* 0157:H7, have the ability to remodel the actin cytoskeleton of infected cells, producing so-called “attaching and effacing” (AE) lesions in the host cell. A 35.4- kb pathogenicity island in enteropathic *E. coli*, called the locus of enterocyte effacement (LEE), has been found to contain all the known genes necessary for AE lesions [119]. Several features of the LEE suggest that it has been acquired recently by the enteropathogenic *E. coli* and other AE bacteria. These include

- (i) a G+ C content of 38.4%, compared with the 51% of the bacterial chromosome as a whole
- (ii) the strict conservation of LEE base sequences within AE pathogens of three genera of bacteria, including the distantly related *Hafnia alvei*, and
- (iii) insertion within *selC* tRNA gene, the same site of insertion for *pai I* in uropathogenic *E. coli* and the bacteriophage ϕ R73.

The LEE is one among a whole class of genetic systems (type III systems) that are extremely sophisticated in exploiting host cell functions (see below).

9.2 Exploitation of host cell functions by bacterial pathogens

Recent studies reveal the common strategies used by a wide range of pathogenic bacteria as well as the unique tactics adopted by individual species in establishing infection. Many host cell functions are exploited by the pathogens, including signal transduction pathways and rearrangements of the cytoskeleton [115]. (The cytoskeleton is literally the skeleton of the cell, composed of special proteins.)

Adhesion to host cells is the first stage in disease development. Bacterial pathogens produce a range of proteins, adhesins, which mediate adhesion to host cells. These adhesins interact with surface membrane components of the mammalian cell, many of which serve as receptors for the bacterial adhesins. The host cell is often an active participant in adhesion by synthesizing the receptors for adhesion. For example, enteropathogenic *E. coli* adheres to intestinal epithelial cell surfaces by destroying host microvilli (protruding processes on the epithelial cells of the intestine) and rearranging the cytoskeleton to form a pedestal on the host cell surface on which the bacterium resides (the AE lesions). To achieve this, the bacterium secretes at least two proteins, EspA and EspB, which activate host cells by inducing a series of events involved in signal transduction: calcium flux, inositol phosphate production, phosphorylation of the amino-acid tyrosine in a membrane protein, and finally, rearrangement of the cytoskeleton. The secretion of the proteins that activate epithelial cells is mediated by a specialized secretion system called the type III secretion system, versions of which are being identified in an ever-increasing number of human, animal and plant pathogens. They allow the bacteria to attach to cells, to enter the cells, or to prevent themselves from being swallowed by phagocytic cells.

Type III secretion systems comprise at least 20 gene products which are essential for the virulence of these pathogens; the genes encode both secreted effector proteins and the machinery necessary for secretion and translocation into target cells. Enhanced secretion of the virulence factors often occurs after contact with host cell surfaces. Although the secreted virulence factors differ among pathogens which cause different diseases, the secretion machinery is often interchangeable. The same type III system is involved in invading cells by activating host signalling pathways, leading to appropriate changes in the cytoskeleton which internalize the pathogens. One type of internalization mechanism is shared by *Yersinia* and *Listeria*, while another is shared by *Salmonella* and *Shigella*. In *Salmonella*, the type III secretion system responsible for invading host cells forms a pathogenicity island on the chromosome, while in *Shigella*, the system is found in a large "virulence" plasmid.

In addition to invasion systems for entering epithelial cells, *Yersinia* species have evolved mechanisms to *avoid* uptake by phagocytic cells. The anti-phagocytosis strategy relies on the expression of Yop proteins, which also involves type III secretion systems. One protein, Yop E paralyzes the cellular cytoskeleton, while another, Yop H is a broad-spectrum tyrosine kinase that interrupts phosphotyrosine signaling associated with phagocytosis. Yet another protein, Yop O, has homology to mammalian enzymes, serine and threonine kinases involved in signal transduction, and like Yop H, is targeted at the inner surface of the host cell's plasma membrane.

Among Gram-positive bacteria, *Listeria monocytogenes*, enters mammalian intestinal cells by means of a surface protein, internalin. Internalin contains multiple copies of a 22 amino-acid leucine-rich tandem repeat (LRR), a feature of several eukaryotic proteins that are generally involved in protein-protein interactions. Another surface protein, InlB, belonging to the internalin multigene family and displaying similar LRRs, mediates entry into cultured hepatocytes,

HeLa cells and Chinese Hamster ovarian cells.

Type III secretion systems have also been identified in the plant pathogens *Ralstonia*, *Pseudomonas*, *Xanthomonas* and *Erwinia*, and are involved in their ability to cause diseases [120].

Chapter 10

Has the Frequency of Horizontal Gene Transfers Increased Recently?

The evidence is now overwhelming that horizontal gene transfer has been responsible for both the rapid spread of antibiotic resistance and for the emergence of virulent strains of pathogens in recent years. Have horizontal gene transfers increased in scope and frequency since genetic engineering biotechnology began?

In Chapter 1, we alluded to the apparently accelerated rates in the evolution of drug and antibiotic resistance and of new pathogens since the 1980s. Furthermore, sequence identity or near-identity in both virulence and antibiotic resistance genes from unrelated species indicates that a high proportion of the horizontal gene transfers may be very recent.

A study carried out in 1983 on strains of bacteria isolated between 1917 and 1954 — at least ten years into the antibiotics era, but well before genetic engineering got underway — showed that none of the strains carried any antibiotic resistance [121]. By contrast, it takes one to two years in the post-genetic engineering era for resistance to develop and spread, rendering new antibiotics useless in four years [11, 25]. However, 24% of the strains from the pre-genetic engineering era encode genetic information for the transfer of DNA from one bacterium to another; and from at least 19% of the strains, conjugative plasmids carrying no antibiotic resistance were transferred to the laboratory strain *E. coli* K12 [121]. Thus, while conjugative plasmids and other mechanisms for horizontal gene transfer may predate both the antibiotics and genetic engi-

neering eras, their prevalence and the range of drugs to which they confer resistance have greatly increased, especially since genetic engineering got underway.

It would also be of interest to screen these strains from the pre-genetic engineering era for pathogenicity islands, which will contribute to our understanding of how pathogenicity islands have evolved since.

10.1 Barriers to horizontal gene transfer

The frequency of horizontal gene transfers may therefore be determined, not so much by the actual mechanisms that exist for mediating transfer as by the existence of barriers that act against the process [77, 122, 123]. In bacteria, a major mechanism for limiting horizontal gene transfer is restriction — the destruction, or inactivation of foreign DNA. There are 11 restriction/modification systems in enteric bacteria which fall into three families, *hsdR*, *hsdM* and *hsdS*. The alleles (different variants) of the *hsdR* family are responsible for destroying foreign DNA, while those of the *hsdM* and *hsdS* families are responsible for methylating, thereby protecting DNA molecules that escape restriction and facilitating their recombination into the genome. A second barrier is the mismatch-DNA repair system encoded by the *mutL*, *mutS* and *mutH* genes of *E. coli* and *S. typhimurium*, which severely inhibits recombination of non-homologous DNA. However, another genetic system, the error-prone SOS system, activated under stress, stimulates such recombination. In addition, as mentioned in sections 6.1 and 7.3, there are origins of replication and origins of transfer specific to DNA from different species, so that even if DNA is transferred, it cannot be replicated and maintained, so that the chances of integration into the chromosome would also have been much reduced, unless, of course, the DNA has been manipulated to combine origins of replication from different species.

Another important influence on the frequency of horizontal gene

transfers is the ecology of the microbes concerned, as mentioned in Chapter 5. Stressful conditions appear to reduce the effectiveness of restriction and to encourage recombination [124, 125]. Starving bacteria are also more competent in taking up DNA from the environment, and hence to become transformed [126]. And heat treatment greatly increases the frequency of transformation (section 7.1); while the effects of xenobiotics in the environment on horizontal gene transfer, apart from antibiotics, remain largely unknown (see section 7.4).

10.2 Genetic engineering biotechnology breaks down species barriers

One main contributing factor to the recent increase in the scope and frequency of horizontal gene transfers may be the deliberate acts of genetic engineers to break down species barriers. They do so by constructing a range of chimaeric vectors for cloning and transferring genes. These artificial vectors have the following important characteristics that enhance horizontal gene transfer:

- They are already derived from elements that mediate horizontal gene transfer most effectively.
- Their chimaeric nature means that they possess sequence homologies to DNA from widely different species and their viral pathogens, plasmids and transposons, thus facilitating successful horizontal transfers and recombination.
- They routinely contain antibiotic resistance marker genes enhancing their successful transfer in the presence of antibiotics, either intentionally applied or as xenobiotics in the environment.
- They often have origins of replication and transfer sequences (see section 6.1), all of which facilitate horizontal gene transfer and recombination. In this context, the fact that they are

“crippled”, so that genes for mobility and/or virulence are removed, is irrelevant, as helper functions can be supplied by other viruses, plasmids and mobile genetic elements present in the donor, recipient or a third strain of bacteria. And virulence genes can be regained by recombination.

- It is well known that chimaeric plasmids and viral vectors are subject to structural instabilities which make them more prone to recombine [40]. Vector instability is a continuing problem for genetic engineers and the biotech industry as far as the stability of the transferred genes is concerned. It also increases the probability and scope for unintended, secondary horizontal gene transfers, which have already been directly demonstrated (see section 6.1).
- The now-routine incorporation of strong promoters and enhancers in vectors to boost expression of transgenes is one main cause of structural instability, which is in addition to the instability arising from the attendant metabolic stress to the organism that, again, may increase unintended horizontal gene transfers [127].
- Finally, vectors are designed to escape restriction [128], thereby also enhancing the probability of a successful horizontal gene transfer.

The construction of artificial vectors is fundamental to genetic engineering. Some examples of the vectors used are given in Table A.2 (compiled mainly from [40]) in Appendix II. All classes of genetic elements that mediate horizontal gene transfer have been used in constructing vectors: plasmids, phages, transposons, plus a range of pathogenic plant and animal viruses. As stated in a standard textbook on genetic manipulation, “Many animal viruses have been subjugated as vectors. Virtually every virus that has been studied in any detail and that has a DNA genome or a DNA stage in its replication cycle has been manipulated in this way.” [40, p. 225]

Although different classes of vectors are distinguishable on the basis of the main framework sequence, practically every one of them is chimaeric. Important chimaeric vectors are the shuttle vectors which enable genes to be cloned (multiplied) in *E. coli* and transferred (transfected) into unrelated species in every Kingdom. Similarly, vectors used in manipulating plants and animals typically contain sequences from a range of plant and animal viral pathogens, as well as antibiotic resistance genes, often originating from promiscuous resistance plasmids and transposons. Phage vectors and phasmid vectors (hybrid of phage and plasmid) are also extensively used, and may have special relevance for the evolution of pathogenicity islands in bacterial pathogens (see section 9.1).

Thus, genetic engineering biotechnology has effectively opened up highways for horizontal gene transfer and recombination, where previously, there was only restricted access through narrow, tortuous footpaths. These gene transfer highways connect species in every Domain and Kingdom with the microbial populations via the universal mixing vessel, *E. coli*. We review further circumstantial evidence that artificial gene transfer vectors increase the scope and frequency of horizontal gene transfers.

10.3 Artificial gene transfer vectors increase the frequency of horizontal gene transfers

It is not easy to transfer genes successfully between species as, we have already emphasized, there are barriers to horizontal gene transfers (see section 10.1). That is why, apart from transposons which are promiscuous, such events were relatively rare in our evolutionary past. For example, analyses of 145 nonvertebrate globin gene sequences showed that there were probably two cases of horizontal gene transfer: one from the common ancestor of ciliates and the green algae to the ancestor of cyanobacteria; and the other, from the ancestor of the yeasts to the ancestor of bacteria [129]. Natural gene transfer vectors — viruses, plasmids and transposons — have probably always existed, especially in the microbial spe-

cies; but they were to varying degrees host-specific, so that the frequencies of conjugal transfers are higher between the same species than with other species [130]. Naturally isolated vectors — even those that carry antibiotic resistance genes and have therefore already undergone evolution by horizontal gene transfer — have not exhibited the same wide range of transfer capabilities.

Horizontal gene transfers have been directly demonstrated between bacteria in the marine environment [30, 131, 132], in the freshwater environment [133] and in the soil [134]. Again, in all the experiments, horizontal gene transfers were mediated by specially constructed hybrid plasmid vectors, of the sort used in genetic engineering. Horizontal gene transfer occurs preferentially in interfaces between air and water and in the sediment, and especially under nutrient depletion conditions [135], thus refuting the claim that nutrient-rich media are necessary to support horizontal gene transfer. Horizontal gene transfer of antibiotic resistances has even been demonstrated in wastewater treatment ponds, the effluent from which is increasingly being used for irrigation in developing countries [136]. As pointed out in section 7.1, frequencies of horizontal gene transfer may be greater under natural conditions than in the laboratory [93].

Horizontal gene transfer is not limited to the external environment. It has been demonstrated between gut bacteria in mice and chickens [137, 138] and in the gut [139, 140] as well as urogenital and respiratory tract of human beings [141].

Stephenson and Warnes [142] wrote, “The threat of horizontal gene transfer from recombinant organisms to indigenous ones is ...very real and mechanisms exist whereby, at least theoretically, any genetically engineered trait can be transferred to any prokaryotic organism and many eukaryotic ones.” (p. 5)

A year later, another molecular geneticist, who works on transgenic plants, admitted that, “... the potential for horizontal [gene] transfer may be greater than thought previously” [143].

Chapter 11

Biological Containment May be Ineffective

When Paul Berg and others [144] published their letter on “Potential biohazards of recombinant DNA molecules” in *Science*, the main areas of concern were the possibilities of the survival and spread of genetically engineered microorganisms (GEMs) and the dissemination of the new recombinant gene sequences to bacterial hosts throughout the different bacterial communities via horizontal gene transfer [34]. To address those concerns and to comply with their own recommendations, general risk assessments were made on the survival and spread of GEMs and the stability of isolated DNA. However, those assessments were often based on little more than plausible assumptions and not on experimental results.

One assumption was that because GEMs carried genetically engineered plasmids, they would not survive due to their extra burden of genetic information [145]. Moreover, laboratory strains were “biologically contained” due to crippling mutations, which meant that they required specific nutrients for survival. Consequently, the general perception was that even if the GEMs were accidentally released, they would not survive in the environment, and would therefore have no negative impacts. Similarly, released DNA would be rapidly degraded by DNAses in the environment and in the digestive tract, if ingested.

These “impact assessments” made it unnecessary to investigate the actual survival of GEMs in the environment, and allowed genetic engineering biotechnology to proceed without extensive safety

measures. The same arguments were later used to promote relaxation of the already lax safety guidelines. And further relaxations are now being contemplated (see section 11.7).

It is clear that the current “risk assessment” is strongly based on assumptions rather than scientific data. These assumptions, furthermore, can be totally at odds with reality, as they are now revealed to be. This is why such risk assessments are a source of hazard in themselves. “Risk” is technically the probability of a hazardous event multiplied by the hazard caused. If the hazard is great, even a “very low probability” will give significant, unacceptable risk. This is a proper application of the precautionary principle. It is often claimed that the hazards are unknown. That is not so. We have identified at least some of the hazards in the spread of virulence, drug and antibiotic resistance in the recent resurgence of infectious diseases.

Our limited enquiry leads us to conclude that the original concerns of Paul Berg and other scientists on the hazards of genetic engineering may have been borne out by scientific evidence that has accumulated since: horizontal gene transfer (and recombination) is the greatest threat to public health facing us to-day, especially if commercial-scale genetic engineering biotechnology is allowed to continue unchecked. We may only be seeing the tip of the iceberg, as far as the possible contribution of genetic engineering biotechnology to the etiology of infectious diseases is concerned.

In this Chapter, we shall review other relevant findings, indicating that microorganisms genetically engineered for “contained use” may not have been effectively contained.

11.1 DNA can persist in the environment

Genes carried by vectors as naked DNA as well as chromosomal DNA can survive indefinitely, especially when adsorbed to solid particles in all kinds of environment: on surfaces of water, as sedi-

ment, in the soil, where they are efficiently taken up by other microbes (reviewed in [34, 146], see section 7.1). Although DNA is rapidly broken down in waste water, adsorption to solid particles in the sludge, which happens very quickly, will stabilize it and prolong its transforming capacity. In addition, DNA is now found to persist for long periods in the laboratory, clinging to many different surfaces. As already pointed out in section 7.1, transformation by the uptake of DNA is a major route of horizontal gene transfers in the environment. DNA is not only released when cells die, but is actively secreted by living cells, and even fragments of genes can have significant effects when transferred (section 7.1).

As increasing amounts of recombinant DNA, especially of the “naked” variety, are discharged into the environment, the risks associated with DNA transformation will rise exponentially.

11.2 DNA can resist digestion in the gut

Earlier studies supported the assumption that DNA is broken down into small fragments in the stomach and subsequently further degraded by nucleases in the digestive system. But more recently, Schubbert and co-workers [147], using the much more sensitive PCR (polymerase chain reaction) technique, were able to detect large fragments of the M13 phage DNA, which had been fed to mice, in the faeces, the bloodstream and within white blood cells. The persistence of DNA in the gastrointestinal tract suggests that transformation of gut bacteria may occur. This is very relevant to the spread of antibiotic resistance marker genes which are currently found in nearly all commercially approved transgenic food crops.

Subsequent studies by the same group revealed that the DNA fed to mice was taken up, not only by white blood cells, but also by spleen and liver cells. The M13 phage DNA was reisolated from spleen cells and some of it were found to be attached to mouse DNA [148, 149], suggesting that it had become integrated. This raises general questions on the ability of gene transfer vectors to

invade mammalian cells directly, and indeed, such invasions have been known to occur.

11.3 Invasion of mammalian cells by vector DNA and viral DNA

Studies made since the 1970s have documented the ability of bacterial plasmids carrying a mammalian SV40 virus (originally specific to monkeys) to infect cultured mammalian cells, which then proceeded to synthesize the virus [87]. Similarly, bacterial viruses and baculoviruses (attacking insects) can also be taken up by mammalian cells [88, 150]. One main route for the uptake of DNA by mammalian cells is by phagocytosis of bacteria containing the vector DNA. But the baculovirus can gain access by itself. It is so good at getting into human liver cells that it is being engineered as a vector for gene replacement therapy [88, 150] — at the same time that it is being engineered in agriculture to be a more effective insecticide. This should raise serious safety concerns [127].

An important route for horizontal gene transfer from bacteria to mammalian cells may be conjugation. Bacterial conjugative plasmids can mobilize DNA transfer between bacteria and yeast (see section 7.3). It was predicted that these plasmids raise the possibility that “conjugation could occur between bacteria and animal cells and between bacteria and plant cells” [71, p. 209]. This prediction has been borne out. Recently, the prokaryote-eukaryote shuttle vectors pAT497 and pAT498 were both transferred from *E. coli* cells into mammalian cells by conjugation, with a helper plasmid from *Shigella* [151].

Once inside cells, the DNA may integrate at random into the host cell genome, causing correspondingly random genetic disturbances. Of particular concerns in this regard are vector DNA engineered with oncogenes (genes associated with cancer development) and other viral sequences which can cause cancers. Recombination between vector DNA and endogenous viruses can also generate new

viral pathogens implicated in several types of animal cancers [35, Chapter 13].

11.4 “Crippled” laboratory strains of bacteria can survive in the environment

There are a number of studies documenting the survival of “crippled” laboratory strains of bacteria in the environment. *E. coli* K12 has been reisolated from lab coats after 20 days [152]. Dott *et al.* [153] inoculated six different strains of GEMs into drinking water, surface water, sewage water and different kinds of soil. On introduction into the sewage under aerobic conditions, the GEMs died off quickly, but not under anaerobic conditions, where only one strain failed to survive the test period of 31 days. However, in sandy soil and nutrient-rich garden soil, the GEM that survived least well in any kind of water had the longest life span.

Survival rates estimated by routine culture methods may be quite misleading. It is now well known that all bacteria can survive starvation and undergo “adaptive mutations” when presented with substrates that they cannot use initially (see Chapter 5). Furthermore, bacteria can survive indefinitely in a non-proliferating, dormant form. This has been documented for a laboratory strain of *E. coli* K12 which, when introduced into the sewage, went dormant and undetectable for 12 days before reappearing, having acquired a new plasmid for multidrug resistance that enabled it to compete with the naturally occurring bacteria [25]. Dormant forms of bacteria and viruses can survive indefinitely as biofilms made up of extracellular matrices containing communities of single or multiple species. These are organized assemblages which can be found in the circulatory system of our body as well as in the environment [60, 61]. In this state, the bacteria continue to accumulate new mutations, to exchange genes, and to come back with a vengeance, when the ecological conditions are ripe.

11.5 Direct documentation of the acquisition of antibiotic resistance, its spread by horizontal gene transfer and persistence

A study in the former East Germany [25] gives an idea of the rapidity with which antibiotic resistance can develop and spread by horizontal gene transfer, and yet persist after the antibiotic ceased to be administered. The antibiotic streptothricin was administered to pigs beginning in 1982. By 1983, plasmids encoding streptothricin resistance had been found in the pig gut bacteria. These had spread to the gut bacteria of farmworkers and their family members by 1984, and to the general public and pathological strains of bacteria the following year. The antibiotic was withdrawn in 1990. Yet the prevalence of the resistance plasmid remained high when monitored in 1993, confirming the ability of microbial populations to serve as stable reservoirs for replication, recombination and horizontal gene transfer, in the absence of “selective pressure”.

In a direct test of persistence of streptomycin resistance [154], researchers cultured many independent lines of a streptomycin-resistant mutant of *E. coli* in the *absence* of the antibiotic. They found that all retained the resistance after 180 generations. Furthermore, the lines had, in the meantime, accumulated compensatory mutations in other parts of the genome which increased their competitive ability relative to the wild type. Again, this demonstrates the irrelevance of conventional explanations of the “natural selection of random mutations” with regard to the evolution of antibiotic resistance.

11.6 Chemical inactivation may be ineffective

The above findings take on additional significance in the light of a recent report that the chemical treatment in waste-tanks of commercial aircrafts is insufficient to inactivate up to 10% of viral and bacterial pathogens [39]. Commercial-scale contained users of GEMs routinely release large amounts of wastes after chemical or physical “inactivation”. How adequate are these inactivation meas-

ures? Even if inactivation is effective, we now know that the large amounts of recombinant DNA released can still be readily transferred to other bacteria by direct uptake (i.e., transformation). What is the current status of safety regulations in the light of all the recent findings?

11.7 Safety regulations are grossly inadequate, and yet further relaxation is contemplated

Novo Nordisk is a biotech company that prides itself on being environmentally conscious and transparent in its safety practices. Their 1996 Environmental Report (kindly sent to Mae-Wan Ho by the Environment Director) gives the following limits — set by the Danish authority — for untreated release of GEMs in terms of colony-forming units (roughly equivalent to live bacteria): waste water, 10,000/ml, air 10,000/ml, solid waste, 10,000/g. These “tolerated releases” have to be seen in the light of the minimum infective dose for *E. coli* 0157:H7, which is less than 50 bacteria [4]. The same company also recycles inactivated GEMs as fertilizers for crops, under the trade name NovoGro.*

* M.W.H. wrote to Lise Kingo, Novo Nordisk’s Director of Environment on 30 June 1997, expressing her reservations on existing practice and regulations on contained use, requesting more information on their monitoring of released GEMs and whether it included monitoring for horizontal gene transfer. Kingo replied by e-mail two weeks later, stating that their scientists were on holiday. M.W.H. raised the matter again, during the UN Biosafety meeting in Montreal in October 1997, with Mr. J.L. Mahler, Novo Nordisk’s Vice President of External Affairs, who also attended the meeting. Mr. Mahler agreed to remind their scientists to send a reply. This came from Lise Kingo more than two months later, stating that horizontal gene transfer is nothing new. “It is a well known and naturally occurring phenomenon which was taken into account by legislators both when the present contained use directive was written and when its current revision was proposed.” Therefore, they saw no need to review existing practice and regulations. Again, they failed to answer M.W.H.’s questions on monitoring for GEM survival and for horizontal gene transfer.

Even if chemical inactivation methods are 100% effective, large amounts of recombinant DNA have already been, and continue to be, routinely released into the environment in one form or another, either in the sewage or directly spread onto fields. Many kinds of dangerous recombinant DNA, containing pathogenic sequences, cancer-causing viruses, as well as antibiotic resistance genes can almost certainly transform bacteria in the environment and further recombine, spreading not only antibiotic resistance and virulence genes in bacteria, but also generating new viral pathogens.

There is an urgent need to reassess the safety regulations, not only of deliberate releases, but also of contained use. Instead of which, our regulators are contemplating further relaxation.

The UK Health and Safety Executive (HSE) has drafted a document, circulated for consultation in June 1997, which would allow commercial and other contained users to release certain classes of live GEMs as liquid wastes into the environment, without dilution or chemical inactivation.* In September the same year, the US Environmental Protection Agency (EPA) authorized a biotech company to sell 500,000 lb of a genetically engineered *Rhizobium meliloti* strain, for coating alfalfa seeds before planting. The GE *Rhizobium*

* The consultation document, Draft Guidance on Certificate of Exemption No. 1, was not widely circulated even within the genetic engineering community. After several exchanges of correspondence between M.W.H. and members of parliament, the Health and Safety Executive agreed, in a letter dated 10 October 1997, that "some important issues" were raised, which were to be discussed by the Technical Sub-Committee (TSC) of the Advisory Committee for Genetic Manipulation. The result of the deliberation was communicated to M.W.H. in a letter dated 17 December, which stated,

"The Committee felt that some of the issues that had been raised went wider than just the hazards associated with any possible discharge of Group 1 GMMs [genetically modified microorganisms] and there were implications for the disposal of both inactivated and non-inactivated waste...

"contains an extra copy of the gene controlling nitrogenase production, genes to increase internal energy production, a gene conferring resistance to the antibiotics streptomycin and spectinomycin, and some uncharacterized DNA"" The antibiotics are currently used in treating TB, the top killer in the world today. This news item was put out on the internet for some time by one of the EPA's own officials, who has been protesting against the agency's effective lack of biosafety oversight on genetically engineered products.

"While the issues you raised are not new, we agree that they are important. We believe that most aspects are already covered sufficiently in the current guidance, however, some of the issues discussed perhaps warrant further attention as part of our continuing programme of update in the light of scientific evidence. HSE has decided therefore to commission an independent, thorough and critical review of the literature, in particular focusing on any new data available on the hazards which may be associated with gene transfer from GMMs in the environment and the hazards associated with free DNA released by such micro-organisms..."

However, we doubt that further literature review is sufficient. We need targeted experimental research on horizontal gene transfer, and DNA sequence comparisons between, say, pathogenicity islands and phage and phasmid vectors, for example.

** "First commercial release of microorganism under TSCA" E-mail message from Suzanne Wuerthele, 22 September 1997.
<Wuerthele.Suzanne@epamail.epa.gov>

Chapter 12

Conclusion

In this paper, we have reviewed a variety of evidence — inductive, deductive and circumstantial — linking genetic engineering biotechnology to the recent resurgence in infectious diseases. A summary of the evidence is presented below.

12.1 Possible links between genetic engineering biotechnology and the recent resurgence of infectious diseases

Inductive

- a. Horizontal gene transfer (and consequent recombination) is found to be responsible for the spread of virulence and antibiotic resistance.
- b. Direct experimental evidence of horizontal gene transfers, some between phylogenetically distant species, has been obtained in all natural environments as well as in the gastrointestinal tract. These were all accomplished using artificially constructed vectors.
- c. Genetic engineering biotechnology makes extensive use of antibiotic resistance genes as selectable markers, thereby increasing the spread of antibiotic resistance genes.
- d. The presence of antibiotics increases the frequency of horizontal gene transfers 10- to 10,000-fold, thereby encouraging

the horizontal transfer and recombination of virulence as well as antibiotic resistance genes.

- e. "Crippled" strains of bacteria can survive in the environment to exchange genes with bacteria in the environment.
- f. DNA released from dead cells (as well as live cells) is not readily broken down in the general environment, nor in the gastrointestinal tract, where it may retain the ability to transform other bacteria.
- g. Some viral DNA are more infectious than the virus itself.
- h. Routine chemical inactivation of genetically engineered microorganisms (GEMs) prior to disposal in the general environment may be ineffective, leaving a substantial proportion of viruses and bacteria in an infective state.
- i. Current legal limits of "tolerated releases" of GEMs from contained use vastly exceed the minimal infective dose of pathogens and potential pathogens.
- j. Non-pathogens are transformed into pathogens by horizontal gene transfer of unit blocks of virulence genes.
- k. Horizontal gene transfers are bi-directional. Released non-pathogens can be readily converted into pathogens in one step, by acquiring unit blocks of virulence genes.

Deductive

- a. Genetic engineering is based on facilitating horizontal gene transfer between distant species by constructing vectors that break down species barriers.
- b. The artificial vectors constructed for genetic engineering are

chimaeric combinations of viral pathogens and other invasive genetic elements that can generate new, cross-species viral pathogens.

- c. The artificial vectors constructed for genetic engineering are inherently unstable and prone to recombination, thereby enhancing horizontal gene transfer and recombination.
- d. Shuttle vectors made by genetic engineering are essentially unstoppable, as they contain signals for transfer and replication in different species; and helper functions for mobilization and transfer can be supplied by viruses, plasmids and transposons which occur naturally in bacteria in all environments.

Circumstantial

- a. The accelerated emergence of infectious diseases and of drug and antibiotic resistance coincides with the development of commercial genetic engineering biotechnology.
- b. Many horizontal gene transfer events responsible for the spread of virulence and antibiotic resistance are recent, based on the high degree (more than 99%) of homologies in sequences of genes found in unrelated species.

The totality of evidence is sufficiently compelling, especially in view of the precautionary principle, to warrant, at the very least, an independent, full public enquiry into genetic engineering biotechnology and the etiology of infectious diseases. In addition, we desperately need research directed at understanding general mechanisms for horizontal gene transfer, which aims to strengthen the barriers against the transfer of recombinant DNA, or which can form the basis for scientific risk assessments. Such research must be carried out by independent research groups dedicated to the task, and not left in the hands of those who are involved in commercial exploitation of genetic engineering biotechnology.

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Appendix I. Genetics of active efflux antibiotic resistance systems

Table A.1 Examples of active efflux systems in bacteria

Transporter	Organism	Antibiotics
	1a. Major facilitator in Gram-positive bacteria*	
<i>OtrB</i>	<i>Streptomyces rimosus</i>	Oxytetracycline
<i>Tet(L)</i>	<i>Bacillus subtilis</i>	Tetracycline
	various cocci	
<i>Mmr</i>	<i>Streptomyces coelicolor</i>	Methylenomycin
<i>ActII</i>	<i>S. coelicolor</i>	Actinorhodin
<i>TcmA</i>	<i>Staphylococcus glaucescens</i>	Tetracenomycin
<i>NorA</i>	<i>Staphylococcus aureus</i>	Fluoroquinolones, basic dyes, puromycin, chloramphenicol, tetraphenylphosphonium
<i>QacA</i>	<i>S. aureus</i>	Quaternary ammonium compounds, basic dyes
<i>Bmr</i>	<i>B. subtilis</i>	Basic dyes, chloramphenicol, puromycin, fluoroquinolones
	1b. Major facilitator in Gram-negative bacteria*	
<i>TetA</i>	<i>E. coli</i>	Tetracycline
<i>CmlA</i>	<i>Pseudomonas aeruginosa</i>	Chloramphenicol
<i>Bcr</i>	<i>E. coli</i>	Bicyclomycin
<i>EmrB</i>	<i>E. coli</i>	CCCP, nalidixic acid, tetrachlorosalicylanilide, phenylmercury acetate

<i>EmrD</i>	<i>E. coli</i>	CCCP, phenylmercury acetate
2. <i>RND</i> family		
<i>AcreE</i>	<i>E. coli</i>	Basic dyes, SDS, erythromycin, novobiocin, fusidic acid, tetracycline, mitomycin C
<i>EnvD</i>	<i>E. coli</i>	As above, plus others
<i>MexB</i>	<i>P. aeruginosa</i>	Tetracycline, chloramphenicol, fluoroquinolones, β -lactams, pyoverdine
3. <i>Smr</i> family		
<i>Smr</i>	<i>S. aureus</i>	Quarternary ammonium compounds, basic dyes
<i>QacE</i>	<i>Klebsiella aerogenes</i>	As above
<i>MtrC</i>	<i>E. coli</i>	Methyltriphenylphosphonium, basic dyes.
4. ABC transporters		
<i>MsrA</i>	<i>Staphylococcus epidermidis</i>	14- and 15-membered macrolides
<i>DrrA, B</i>	<i>Streptomyces peucetius</i>	Daunorubicin, doxorubicin
<i>TlrC, B</i>	<i>Streptomyces fradiae</i>	Tylosin

* Gram-positive and -negative bacteria are the traditional major divisions of bacteria. The Gram-positive bacteria are surrounded by a thick peptidoglycan cell wall which, however, is permeable to small molecules; Gram-negative bacteria, such as *E. coli*, are surrounded by a second membrane, which functions as an effective barrier to small molecules.

CCCP, carbonyl cyanide m-chlorophenylhydrazone, SDS, sodium dodecylsulphate

Appendix II. Artificial vectors used in genetic engineering

Table A.2. Examples of vectors constructed for genetic manipulations

Vectors	Sources	Host(s)	Uses
Plasmid vectors			
pBR322	RSF2124 (Col E1, with transposon carrying Ap ^R) pSC101 (with Tc ^R) pMB1 (Col E1-like)	<i>E. coli</i>	Cloning and expressing genes of all species in <i>E. coli</i>
pBR322 shuttles	pBR322 plus various plasmid/viral sequences specific to other species	<i>E. coli</i> and others	Cloning and transfection
pHV14	pBR322 pC194 (CmR plasmid from <i>S. aureus</i>)	<i>E. coli</i> and <i>B. subtilis</i> <i>S. aureus</i> ?	Cloning in <i>B. subtilis</i>
YEp	<i>E. coli</i> plasmids and yeast plasmids	<i>E. coli</i> and yeast	Cloning and transfection
YCp	<i>E. coli</i> plasmids and yeast centromeres	<i>E. coli</i> and yeast	Cloning and transformation
YACs	<i>E. coli</i> plasmids and yeast telomeres	<i>E. coli</i> and yeast	Cloning of large human genome fragments

Bin19 (used with pAL4404)	pPK252 (Group P, with KanR) polylinker, T-DNA of <i>Agrobacterium Ti</i> plasmid with <i>neo</i> marker insert	<i>E. coli</i> and others	Tranformation of plants
pJP181 (used with pAL4404)	pRSF1010 (Group Q, CmR) <i>neo</i> marker	<i>E. coli</i> and others	Transformation of plants
pRSV	pBR322 SV40, bacterial <i>neo</i> or XGPRT marker or mouse DHFR insert Rous sarcoma virus promoter	<i>E. coli</i> and others	Transgene expression in animals
Phage vectors λ gt11	λ phage with <i>E. coli lac</i>	<i>E. coli</i>	Cloning and expression
λ gtWES.T5622	λ phage , phage T5	<i>E. coli</i>	
Cosmid vectors c2XB	<i>cos</i> fragment from λ phage RSF2124 Ap ^R	<i>E. coli</i>	Cloning
Phasmid vectors λ ZAP	Phages λ , M13, T3, T7, <i>E. coli lac</i> , RSF2124 Ap ^R	<i>E. coli</i>	Cloning and expresssion, restriction mapping sequencing

Broad host-range plasmid vectors

KT230	RSF1010 (group Qm, SuR, SmR,) added KmR	<i>E. coli</i> and others	Cloning in other bacteria
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pRK203	RK2 with Km ^R cloned into Col E1	<i>E. coli</i> and others	Mobilize plasmids into other hosts
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Broad host-range transposon pNJ5073	vectors Tn7 (transposon with Tp ^R and Sm ^R) <i>E. coli trpE</i> RK1 (plasmid with Tc ^R)	<i>E. coli</i> and others	Cloning in other bacteria
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Plant viral vectors

CaMV	gene VI and its strong 35S promoter used routinely in vectors for plants	<i>E. coli</i> and others	Boost Transgene expression in plants
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Animal viral vectors

Herpes simplex virus	<i>tk</i> gene incorporated into a range of vectors for animal cells	<i>E. coli</i> , humans, others	Selectable marker
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SV40	hybrid vector with <i>E. coli</i> plasmid promoter and enhancer genes in a range of vectors	<i>E. coli</i> , monkey, others	Tranformation and transgene express in animal cells
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Rous Sarcoma virus	promoter incorporated into a range of vectors for animal cells	<i>E. coli</i> , chicken, others	Transgene expression in animal cells
Bovine papilloma virus	hybrid vector with <i>E. coli</i> plasmid	<i>E. coli</i> , bovine, others	Transformation of animal cells
MMTV	promoter incorporated into a range of vectors	<i>E. coli</i> , mouse	Expression
Vaccinia virus	many recombinant viruses with e.g. hepatitis, AIDS, HTLV. rabies antigen gene	<i>E. coli</i> , humans, others	Vaccines
Baculovirus	many recombinant viruses with toxins against insects recombinant viruses with human genes	<i>E. coli</i> , insects humans	Insecticidal Somatic gene therapy
Adenovirus	recombinant viruses with human genes	<i>E. coli</i> , humans	Somatic gene therapy
Cytomegalovirus	promoter gene in a range of vectors	<i>E. coli</i> , humans	Transfection transformation

Retroviral vectors	hybrid vectors with <i>E. coli</i> plasmid <i>neo</i> marker	<i>E. coli</i> , animals	Transformation and transgene expression
MuLV	broadened host- range hybrid vector	<i>E. coli</i> , mammals, birds	
Transposons as vectors and related uses			
<i>P</i> element	with polylinker	<i>Drosophila</i>	Transformation
	with <i>E. coli ori</i>	<i>E. coli</i>	Mutagenesis
		<i>Drosophila</i>	
	with <i>E. coli lacZ</i> , AmpR	<i>E. coli</i> <i>Drosophila</i>	Enhancer trapping

GLOSSARY

Adaptive mutation, or Directed mutation The phenomenon whereby bacteria and yeast cells in stationary (non-growing) phase have some way of producing (or selectively retaining) only the most appropriate mutations that enable them to make use of new substrates for growth.

Allele A particular variant of a gene.

Amino acid An organic acid carrying an amino group ($-NH_2$). There are 20 kinds of amino acids, which are joined together in a defined order to make up linear molecules of proteins, each of which contains hundreds of amino acids.

Antibiotic A chemical agent that prevents bacteria from proliferating.

ATP, adenosine tri-phosphate A chemical substance present in all organisms whose function is to act as an intermediate for energy transformation.

Bacteriophage Any virus that infects bacteria, also known as **phage**.

Baculovirus A virus that normally infects insects.

Base An organic base joined together with a sugar and a phosphate group to make a nucleotide. Many nucleotides — thousands to millions or more — are linked together through the sugar of one nucleotide to the phosphate of the next to make up long linear molecules called DNA, or RNA. There are four different bases in DNA: adenine, thymine, guanine and cytosine; in RNA, thymine is replaced by uracil.

Base sequence The sequence in which the four different bases occur in a DNA or RNA molecule.

Biofilm A layer of extracellular matrix containing quiescent, non-proliferating microorganisms.

Chromosome A structural unit of genetic material consisting of a long molecule of DNA complexed with special proteins in eukaryotes, but not in prokaryotes

Clone An identical copy of an individual or a gene, or the totality of all the identical copies made from an individual or a gene. In genetics, the clone is identical in genetic make-up to the original.

Conjugation The mating process in bacteria which requires cell-to-cell contact being established, and in which genes are transferred between cells.

Directed mutation See **Adaptive mutation**.

DNA Deoxyribonucleic acid, the genetic material consisting of a long chain of individual units called nucleotides, each consisting of a base joined to a sugar and a phosphate group. There are four different bases, guanine (G), cytosine (C), thymine (T) and adenine (A). The precise sequence of bases in the chain is different for different genetic material. The DNA molecule usually exists as a complex of two chains or strands in an intertwined double helix, in which the bases are paired in complementary fashion: G with C and T with A. The strand that codes for a polypeptide is the sense strand. Its complement is the antisense strand.

DNase An enzyme that breaks down DNA.

DNA methylation An endogenous process in the cell which adds a methyl group, $-CH_3$, to the base cytosine or adenosine, resulting in gene silencing, or failure of the gene to become expressed.

DNA polymerase An enzyme that makes DNA.

Dominant allele An **allele** which is expressed when only one copy is present in an individual, i.e., in heterozygous condition.

Ecosystem The totality of all plant and animal species that constitute an interdependent, interrelated community.

Enzyme A protein produced by living organisms that acts as a catalyst for a specific biochemical (metabolic) reaction.

Epistasis Interaction between genes.

Etiology The science of causes.

Eukaryote The major class of living things, including all multicellular, higher organisms and some single-celled organisms, that have a **nucleus** in their cells, containing the chromosomes.

GEMs or GMMs Genetically engineered microorganisms or, equivalently, genetically modified microorganisms.

Gene A unit of heredity, usually a stretch of DNA with a well-defined function, such as one coding for a protein, or one that promotes transcription of other proteins.

Gene amplification The process whereby genes or a sequence of DNA in the genome is greatly increased in number of copies.

Gene cloning The technique of making many copies of a gene, isolating the gene and identifying it.

Gene expression In molecular genetics, this usually means the eventual appearance of the polypeptide encoded by the gene.

Gene silencing The process(es) whereby certain genes in the genome are prevented from being expressed by chemical modifications and other means.

Gene replacement therapy Treating diseases by replacing the defective gene, either by incorporating a normal copy of the gene in the germ-cells (egg or sperm) or in the embryo (germline gene replacement therapy), or by supplying copies of the normal gene to be taken up and incorporated into cells of the adult (somatic cell gene replacement therapy).

Genetic code The code establishing the correspondence between the sequence of bases in nucleic acids (DNA and the complementary RNA) and the sequence of amino acids in proteins.

Genetic engineering The manipulating of genetic material in the laboratory. It includes isolating, copying, and multiplying genes, recombining genes or DNA from different species, and transferring genes from one species to another, bypassing the reproductive process.

Genetic marker Any segment of DNA that can be identified, or whose chromosomal location is known, so that it can be used as a reference point to map or locate other genes. Any gene which has an identifiable phenotype that can be used to track the presence or absence of other genes on the same piece of DNA transferred into a cell.

Genome The totality of the genetic material of a cell or organism.

Genotype The precise variant (s) of the gene (s) carried by an individual.

Heterozygous A condition in which two different alleles of the gene are present in an individual, as opposed to homozygous, in which two copies of the same allele are present.

Horizontal gene transfer Transfer of genes from one individual to another, of the same or different species, usually by means other than cross-breeding.

Integron A special kind of mobile genetic element that allows insertion of antibiotic-resistant genes into specific sites, each of which is provided with a promoter for expression. Integrons can jump into and out of the bacterial chromosome and of plasmids, and are particularly involved in the evolution of multiple resistances to drugs and antibiotics.

Interrupted genes Genes whose coding sequence is interrupted at intervals by long stretches of non-coding sequences. The coding regions came to be known as exons and the non-coding regions as introns. This structure is now found to be characteristic of most eukaryotic genes. The number and size of introns vary greatly, and they are often much longer than the coding sequences. After transcription, the intron regions are removed, or spliced out, from the RNA transcript before it is translated into protein.

Intron A non-coding region in a gene, which comes between exons, the coding regions.

Messenger RNA (mRNA) The RNA intermediate in protein synthesis containing a transcribed copy of the gene sequence that specifies the amino-acid sequence of the polypeptide it encodes.

Metabolism The sum total of the chemical processes that take place in living organisms, resulting in growth, development, and all other forms of energy transformation.

Metabolite One particular chemical intermediate generated in metabolism.

Mitochondria Membrane-bound cellular organelles in which organic substrates derived from food are oxidized to provide energy for all kinds of vital activities. They carry their own complement of DNA and are replicated independently so that when the cell divides, each daughter cell will receive half of the mitochondria.

Mobile Genetic Element, also called **Transposon** or Transposable Genetic Element A sequence of DNA that can move (transpose) from one place to another in the genome of a cell.

Multigene families are genes that exist in multiple copies in the genome, from several copies to many thousands or hundreds of thousands of copies.

Mutagen A substance or agent that causes genetic mutations, or chemical alteration of the genetic material, DNA.

Mutation A chemical alteration in the genetic material, changing the base sequence of the DNA.

Nucleus A structure in the eukaryote cell, bounded by a membrane, that contains the genetic material in the form of DNA organized into chromosomes.

Oncogenes Genes associated with cancer.

Operon A multigenic unit consisting of a regulator gene, a promoter-operator region and one or more other genes whose expression is regulated.

Pathogen Any agent that can cause disease.

Pathogenicity islands Large chromosomal regions in pathogenic bacteria that encode virulence genes acquired by the bacteria from unrelated organisms.

Phage A bacterial virus.

Phenotype The expressed characteristics, or an expressed character of an organism, due to its genotype.

Plasmid A piece of parasitic genetic material found in a cell that can propagate itself using the cell's energetic resources.

Polypeptide or **Protein** A long chain of different amino acids joined together by special chemical (peptide) bonds.

Precautionary principle An internationally agreed principle for policy decisions with regard to protecting the environment and human health: When an activity raises threats of harm to human health or the environment, precautionary measures should be taken even if some cause-and-effect relationships are not fully established scientifically.

Prokaryote The class of living things, including all bacteria, that do not have a nucleus in their cell.

Promoter A stretch of DNA which is required for gene expression. It is the region of a gene at which the RNA polymerase binds to start transcription. Most promoters are located upstream of the gene, except some eukaryotic genes which have promoters internal to the gene.

Proto-oncogenes Cellular genes which, when mutated or over-expressed, become **oncogenes**.

Provirus A virus that has inserted its genome or a complementary copy of its genome into the host cell genome.

Recombination The formation of new combinations of alleles or new genes which occur when two homologous DNA or chromosomes break and exchange parts.

Reductionism The doctrine that complex systems can be completely understood in terms of its simplest parts. For example, an organism is to be completely understood in terms of its genes, a society in terms of its individuals, and so on.

Regulator gene A gene that acts to turn other genes on or off.

Regulon A unit containing many operons which are regulated by some environmental stimuli, such as heat, shock, or the presence of light.

Replicon A unit containing many genes which are replicated together.

Repressor A protein that binds to an operator region of another gene to prevent it from being expressed.

Restriction The process whereby DNA is cut with restriction enzyme (s).

Restriction enzyme An enzyme which cuts DNA based on the recognition of a short specific base sequence in the DNA.

Retrotransposon A mobile genetic element that depends on a reverse transcription step to move and to duplicate.

Retrovirus An RNA virus that depends on reverse transcription — making a complementary DNA copy of the viral RNA, which is inserted into the host genome — for its replication. Retroviruses include many cancer viruses and the AIDS virus.

Reverse transcription The reverse of transcription — making a copy of complementary DNA (cDNA) from an RNA sequence — catalyzed by the enzyme, reverse transcriptase.

Ribosome An organelle in the cell required for protein synthesis.

Ribosomal RNA (rRNA) RNA molecules which make up the ribosome.

RNA Ribonucleic acid, similar to DNA except for the sugar in the nucleotide unit, which is ribose, instead of deoxyribose, and the base uracil instead of thymine. RNA is the genetic material for RNA viruses. It is also the product of **transcription**, whereby the sequence of bases in DNA is copied into a complementary sequence of RNA.

RNA polymerase An enzyme that makes RNA.

Selection A process whereby those organisms with a certain trait are selected to survive and reproduce, while the rest are eliminated.

Shuttle vectors Vectors that can replicate and infect distantly related species.

Substrate A chemical substance that takes part in a chemical reaction catalyzed by an enzyme.

T-DNA The tumour-causing part of the Tumour-inducing (*Ti*) plasmid of *Agrobacterium*, which is transferred to the plant host genome during an infection.

Transcription The process of making a complementary sequence of the gene sequence in the genome, which is either used directly, as in case of **ribosomal RNAs** (rRNAs) and **transfer RNAs** (tRNAs), or is further processed into the **messenger RNA**, which is translated into protein. The process is catalyzed by the enzyme known as DNA-dependent RNA polymerase.

Transcription factors Proteins in eukaryotes that regulate the transcription of other genes by binding to regulatory sequences of the gene, the interaction with one another and with the RNA polymerase.

Transduction In genetics, the transfer of genes by viruses from one organism to another.

Transfection The process whereby a foreign gene carried on an artificial vector is used to infect cells.

Transfer RNA (tRNA) RNA molecules which transfer specific amino acids to the **messenger RNA** so that the polypeptide it encodes can be synthesized.

Transformation In genetics, the transfer of genes by one organism taking up DNA belonging to another organism of the same or different species.

Transgenic organism An organism created by genetic engineering, in which one or more foreign genes have been incorporated into its genome.

Translation The step in protein synthesis in which the messenger RNA directs the synthesis of a polypeptide of a particular amino-acid sequence by “decoding” the genetic code.

Transposon See **Mobile genetic element**.

Vector A carrier of genes or of disease. For example, viruses are vectors for genes, whereas the mosquito is a vector for malaria.

Virulence Ability (of pathogens) to infect organisms and cause disease.

Virus A parasitic genetic element enclosed in a protein coat that can replicate in cells, and form infectious particles, or remain dormant in the cells. Its genetic material can become integrated into the cell's genome to form **provirus**.

Xenobiotics Chemical pollutants in the environment which are foreign to living organisms, or which occur in concentrations that are harmful to living organisms.

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