3 Biosafety in rDNA Research and Production

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1 Introduction

Modern biotechnology represents the most exciting advance in the biological sciences this century. Particularly genetic engineering opened up completely new horizons for general basic research in biological systems which subsequently are bound to be followed by new prospects for medical, agricultural and industrial applications. However, biotechnology is not in itself a science, an industry nor a product but a powerful set of tools which is increasingly used to develop processes and to manufacture many products for every-day use or consumption as well as for industrial uses.

Much of the current confusion and lack of policy coherence arises from the tendency to think of biotechnology as fundamentally different from other technologies and therefore requiring special rules particularly with respect to biosafety issues. This equally concerns the handling of recombinant organisms in the laboratory and production facility, consumption or use of products from such processes and the deliberate release of organisms into the environment with the objective of their survival therein. While all regulations of modern biotechnology worldwide differentiate and thereby exclude applications of non-recombinant methods like diagnosis, “in vitro” fertilization or embryo-splitting correctly, the interest groups profoundly hostile to biotechnology continue to scramble up all applications and construct horror scenarios well received by parts of the media.

This chapter will focus on biosafety issues which are relevant to the handling of microorganisms in “contained use” and thereby review the experience accumulated in both microbiology and molecular biology which is the basis for the assessment of biological risks of organisms containing recombinant DNA molecules (the widely used term “recombinant organism” is actually misleading and should only be used for organisms produced by cell-fusion technology). While for “naturally” occurring organisms, and particularly pathogenic microorganisms, much scientific knowledge has accumulated since the end of last century, the “safety issues” of the debate on “recombinant DNA technology” (to be differentiated from the “socio-economic” issues) was and still is largely based on the presumption that recombinant organisms are “unique”. While the scientific community in vain tried to falsify this view, it was taken up by the media and particularly by the European environmental agencies and the European political establishment resulting in regulations specific for the application of recombinant DNA technology instead of more appropriately introducing regulations for operations with pathogenic organisms and biological entities potentially harmful to parts of the environment as well as product approval systems evaluating the safety of biological products primarily by their properties and not by the methods they are manufactured. Consequently, assessment procedures were introduced which allow the comparison of a recombinant organism with its non-recombinant wild type or comparable non-recombinant organisms. This leads to a classification of the recombinant organism either to be harmless or to be classified into one out of the three risk groups (three is the highest risk group) which are used for the classification of naturally occurring pathogenic microorganisms.

Safety issues related to food and pharmaceutical products and the deliberate release of GMOs are treated in different chapters in this volume.

2 The Development of Molecular Biology

Biotechnology is best characterized as an interdisciplinary issue and can be defined as follows (SAGB, 1990):

“Biotechnology” refers to the application of living organisms and their cellular, subcellular or molecular components to create products and processes.

Modern biotechnology, and in particular genetic engineering, stems from knowledge and methods developed in different areas of research like microbiology (bacteriology and
virology), cell biology, biochemistry and other specialist fields.

Since Gregor Mendel discovered the basic principles of the laws of heredity in 1866 (published under the title “Experiments with Plant Hybrids”) and Darwin’s discovery of the evolution of organisms, the prime goal of basic research in biological science has been to find out what these hereditary characteristics and their relation to the evolution of organisms actually are and how they work. Ultimately, this scientific quest aims to understand life; at least its biological basis.

Although deoxyribonucleic acid (DNA) was discovered by Miescher long ago in 1869, it still took another 75 years for detecting the functional relationship between DNA and the genetic properties of living organisms by Avery (1944). Once it had been demonstrated that the chromosomes located in the cell nuclei are the carriers of hereditary information, Watson and Crick succeeded in 1953 in making the pioneering discovery that DNA consists of two molecular chains in the form of strands which are arranged in a helical structure as a double strand. Since Jackson, Symons and Berg (1972) first constructed a DNA molecule which was capable of multiplication (“recombinant” plasmid), genetic engineering is the major contributor to the dramatic progress in understanding biological systems in molecular terms and for unique applications from industrial processes to medical therapy.

As the genetic engineering methods can be rapidly understood, learnt and put to practical application using excellent manuals (Ausbébel et al., 1989), they will not be presented here.

3 Determinants of Pathogenicity

Before organisms and the work to be conducted with them are allocated to a safety level, a risk evaluation must be conducted. This implies that it is necessary to define exactly what actually a pathogenic microorganism is: namely an organisms which is known to be able to cause a disease (or diseases) in humans, animals or plants or for which there is a well-founded suspicion that this may be the case. An important subject in this context is the measurement of pathogenicity because, in a strict sense, the term pathogenicity has only a comparative and no absolute meaning. Therefore, because one microbial population can be more pathogenic than another and standard species or strains are not defined (nor seriously can), pathogenicity often can be compared only by inoculating animals with graded doses of the respective strains until a disease is detected. However, species differences, location of (primary) infection and the general health “status” of the animals are difficult to compare. Not surprisingly therefore, reliable methods for growing and quantifying the pathogens or valid animal models often are not available. For the classification of pathogens, some general aspects need to be considered for the evaluation and in this context are:

- The quantification of the inoculum (infectious dosis) which is relatively simple to determine for many bacteria but less satisfactory for viruses and fungi. Additionally, the route of inoculation (e.g., by aerosol or intraperitonial) often affects both the necessary dosage and virulence itself.
- The comparison of pathogenicity between species is almost impossible in quantitative and qualitative terms, when, e.g., the influenza virus causes a respiratory disease in ferrets and the lassa virus produces a hemorrhagic fever in mice or rabbits (these are the standard animal models).
- The ideal test animal is a natural host, which essentially only applies to veterinary diseases. The choice of experimental animals with similar disease syndromes is often difficult to achieve, and primates are usually not available for many studies; while there is no good animal model available for human measles, the aerosol infection of guinea pigs with Legionella pneumophila is a good model of the respective human pneumonia (regarded as zoonosis), and the
oral infection of primates with \textit{Shigella} spp. mimics the human bacillary dysentery (shigellosis).

- The in-depth analysis of the multifactorial nature of pathogenicity, e.g., with respect to mucosal infection and invasion, multiplication in or outside cells, evasion of host defenses like phagocytosis or complement action, immunopathology etc. has progressed much further with bacteria than with viruses, fungi or parasites.

- Pathogenic determinants can be identified by either chemical mutation and biochemical purification or by genetic modification. However, with respect to the isolation of surface markers it must be kept in mind that such markers are not necessarily identical with the determinant(s) of pathogenicity. Only the use of the recently developed genetic engineering methods allows the analysis of individual pathogenic determinants, e.g., by cloning a determinant from a pathogenic strain into \textit{Escherichia coli} and subsequently reintroducing the determinant into a deficient strain thereby eventually reestablishing “natural” pathogenicity. In addition, for the demonstration of its biological significance, the cloned determinant can be easily mutated \textit{in vitro} with a concomitant loss of its biological properties.

During the last 20 to 30 years much knowledge for many pathogenic species has accumulated on the multifactorial nature of particularly bacterial pathogenicity, and numerous components of pathogenicity have been identified and probed. A good example may be the following collection of pathogenic determinants of \textit{Vibrio cholerae}, which may not even be comprehensive (MÜLLER, 1992):

- one heat-labile and one heat-stable toxin
- one hemolysine
- one N-acetylhexose aminase
- one neuraminidase
- one nicotinamide-adenine dinucleotidase
- several proteases
- fucose- and mannose-sensible hemagglutinins
- “outer-membrane” proteins and
- motile flagellae.

Since these pathogenic determinants will not have developed to have pathogenic effects on the human intestine, their natural and ecological functions have to be interpreted with respect to the natural niche of \textit{Vibrio cholerae}, which is the colon and exoskeleton of crab species. This means that this human pathogen may be a harmless facultative or even obligate parasite in lower crustacea (HUQ \textit{et al.}, 1986) and underlines the introductory remark that pathogenicity is not a strict but relative term.

It remains to be seen what the functions of such determinants in their normal niches really are. For legionellas it was found that the normal ecology as well as pathogenicity coincide: Namely the ability to multiply intracellularly within free-living amoebas in the same manner as in human leukocytes within the human lung causing pneumonia. Insofar, listerias and “atypical” mycobacteria may exhibit similar patterns.

With respect to an understanding of pathogenicity it is certainly helpful to distinguish “obligate” pathogens such as \textit{Vibrio cholerae}, \textit{Shigella} spp. or \textit{Salmonella typhi} (each carrying specific pathogenic determinants) from “facultative” pathogenic organisms such as \textit{Pseudomonas aeruginosa}, certain streptococci or \textit{E. coli}. While the presence of the classical pathogenic agents in water, food or even in feces may present a high risk for the community, the latter “intestinal” organisms belong to the natural flora of the human body or to those microorganisms which are typically found in the environment. Furthermore, it must be kept in mind that particularly feces may contain microorganisms which can be highly pathogenic to the host if introduced parentally into the host (e.g., \textit{Clostridium tetani} via injury). These ubiquitous “facultative” pathogenic organisms only rarely develop strains with pathogenic determinants and mostly may cause mild infections only under certain unusual conditions.

Well known are also “opportunistic” entropathogenic bacterial strains which usually
do not cause infections except in, e.g., immunologically compromised HIV patients (Ruf and Pohle, 1989). Ubiquitous microorganisms, such as some “atypical” strains of Mycobacterium, Salmonella, Shigella, Campylobacter, Clostridium, and some fungi, which were reported to use the opportunity to colonize and propagate in the immunocompromised body what they cannot in healthy patients. While these infections usually are accessible to, e.g., antibiotic therapy, infections recur frequently. However, it must be stressed that while such “secondary” infections with opportunistic pathogens can be observed, the often lethal infections in HIV patients are not caused by these opportunistic pathogens but by simultaneous infections with pathogenic organisms (e.g., causing pneumonia or influenza). It is noteworthy to mention, that E. coli, which may cause gastrointestinal infections in healthy patients, are not significant in such opportunistic infections, and that microorganisms such as Saccharomyces cerevisiae, Bacillus subtilis and others, which are classified as non-pathogens, have not been identified as opportunists in these immunocompromised patients.

Last but not least the infectious dose, i.e., the number of pathogenic organisms necessary to establish a disease, is relevant for the classification of pathogenicity, which ranges from about 3 cells for Rickettsia tsutsugamushi or 180 cells for Shigella flexneri to $10^8$ cells for Vibrio cholerae.

For further details concerning issues relevant to pathogenicity, the interested reader is referred to the literature (Smith, 1988; Finlay and Falkow, 1989).

In conclusion, the feasible but difficult task is to classify pathogenicity via the observation of prevalence, duration and severity of the disease produced by the different types of recombinant experiments and also for large-scale uses in 1991. It should be noted that the NIH Guidelines regulate applications with “recombinant molecules” and “organisms containing recombinant DNA molecules” instead of using the rather inaccurate or vague term “genetically modified organism” used by the EU Directives (1990).

As time passed, it became clear that the initial worries about certain risks of rDNA research were greatly overstated, and in particular no evidence has been brought forward until today which would support the early risk scenarios. While this assessment is based on general experience, this view was further supported by experiments designed to explore risks of rDNA experiments (Israel et al., 1979; Chan et al., 1979): In a series of experiments polyoma virus DNA was cloned into bacterial vectors. Neither the recombinant DNA molecules (“naked DNA”) nor E. coli K12 cells carrying these vectors caused viral
### Tab. 1. Vaccines for Infectious Diseases in Humans and Fermenter Volumes Used in Industrial Fermentations

<table>
<thead>
<tr>
<th>Bacterial Diseases</th>
<th>Fermenter Volume (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diphtheria</td>
<td>Toxoid</td>
</tr>
<tr>
<td>Tetanus</td>
<td>Toxoid</td>
</tr>
<tr>
<td>Pertussis</td>
<td>Killed Bordetella pertussis</td>
</tr>
<tr>
<td>Typhoid fever</td>
<td>Killed Salmonella typhi</td>
</tr>
<tr>
<td>Paratyphoid fever</td>
<td>Killed Salmonella paratyphi</td>
</tr>
<tr>
<td>Cholera</td>
<td>Killed/extracts Vibrio cholerae</td>
</tr>
<tr>
<td>Plague</td>
<td>Killed/extracts Yersinia pestis</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>Attenuated Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>Meningitis</td>
<td>Polysaccharide from Neisseria meningitidis</td>
</tr>
<tr>
<td>Bacterial pneumonia</td>
<td>Polysaccharide from Streptococcus pneumoniae</td>
</tr>
<tr>
<td>Typhus fever</td>
<td>Killed Rickettsia prowazeki</td>
</tr>
<tr>
<td>Lepra</td>
<td>Killed Mycobacterium leprae</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Viral Diseases</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Smallpox</td>
<td>Attenuated virus</td>
</tr>
<tr>
<td>Yellow fever</td>
<td>same</td>
</tr>
<tr>
<td>Measles</td>
<td>same</td>
</tr>
<tr>
<td>Mumps</td>
<td>same</td>
</tr>
<tr>
<td>Rubella</td>
<td>same</td>
</tr>
<tr>
<td>Polio</td>
<td>Attenuated (Sabin) or inactivated virus</td>
</tr>
<tr>
<td>Influenza</td>
<td>Attenuated or inactivated virus (Salk)</td>
</tr>
<tr>
<td>Rabies</td>
<td>Inactivated virus</td>
</tr>
</tbody>
</table>

Infections nor tumors in mice. Most significantly, while it has long been known that mammalian cells can take up DNA (Gronenberg et al., 1975), biological effects depend on the nature of the transmitted DNA: baculovirus DNA is not transcribed and rapidly eliminated from mammalian cells (Carbonell and Miller, 1987), while the injection of v-onc DNA into animals (Burns et al., 1991) may induce tumors similar to naturally occurring infections with oncogenic viruses. These observations have led to recommendations for the protection of laboratory personnel to apply certain laboratory practices while handling potentially oncogetic DNA (Advisory Committee on Genetic Modification, United Kingdom, 1990). However, the accumulated knowledge clearly indicates that cloned pathogenic viral DNA bears no increased hazards in comparison to the uncloned viral DNA itself.

Any number of scientific experiments may prove that no general or unique biological risks are associated with rDNA experiments, nevertheless, by definition a final risk in individual cases cannot be excluded. With respect to the classification of rDNA work into Good Laboratory Practice (GLP) or one out of three risk categories, the so-called “additive model” has proved to be a useful and reliable tool for assessing potential risks. This model is applied worldwide and basically assumes that recombinant DNA experiments can be classified by inspecting the properties of the host organism, the organism the DNA fragment is derived from and the vector to be used. In addition to this it is assumed that the introduction of DNA segments from a non-pathogenic organism into another non-pathogenic organism (host) does not result in a pathogenic organism. With respect to the determinants responsible for pathogenicity, as outlined before, this assumption appears both reasonable and practical. Moreover, even the introduction of pathogenic determinants (like the ones from Vibrio cholerae) into a non-pathogenic host such as E. coli K12 or yeast is not sufficient to convert the hosts into pathogenic organisms. On the other hand, it is obvious that the cloning of pathogenic deter-
minants from, e.g., *Yersinia pestis* into *Vibrio cholerae* needs the appropriate safety precautions, firstly because the host is pathogenic, and secondly such a modification may (or may not) increase the pathogenicity of the recombinant host in comparison to the wild-type organism. However, if such a modification would be beneficial to the host, it is highly probable that such a modified organism would have established itself by means of a “natural” gene flow which is known to exist among microorganisms (Lorenz and Wackernagel, 1994).

In practical evaluations, the different biological backgrounds into which a certain gene (or set of genes) is cloned and potential “synergistic effects” originating thereof are not fully predictable in any case but not necessarily new or ignored. At the genetic level, an example for such effects is the activation of recombinational events leading to genomic rearrangements in developing lymphocytes by two adjacent genes (Oettinger et al., 1990). Similar effects are known from a variety of biological systems, e.g., *Drosophila*, the activation of oncogenes (positioning effects) or recombinational events during embryogenesis. Altogether, while such effects may amplify or weaken certain properties of an organism or result in modified properties or substances being synthesized, it is hard to imagine that such effects could convert a non-pathogenic organism into one with unique properties and risks. The vast experience accumulated in rDNA R&D and particularly its excellent safety record support this estimation and champion the additive assessment model as an adequate and pragmatic tool.

In conclusion, the knowledge accumulated in biological and genetic sciences is a sound basis for the evaluation of potential risks associated with the genetic modification of microorganisms. This applies to both classical and recombinant methods used for modifying microorganisms genetically. Beyond the safe handling of non-pathogenic microorganisms, the risk evaluation concerning the use of bacteria, viruses, fungi and parasites which are pathogenic to man principally has to take into account the following criteria:

- Virulence and/or pathogenicity of the organism, i.e., whether in fact an infection is caused and the severity of its course
- Route of infection: aerosols, direct or indirect contact, injuries, vectors, etc.
- Epidemiological behavior: incidence and distribution of a pathogenic organism, immune state of the host organism, role of vectors and possible reservoirs
- Survival conditions in the laboratory
- Ability to survive outside the laboratory/production facility

If a microorganism is classified pathogenic according to the above criteria, several factors should be considered which could be relevant to any kind of work which might change the pathogenic profile and especially increase the pathogenicity of a microorganism. This includes possible effects of the vector(s) being used:

- Production of one or more toxins
- Transfer of genetic elements which have an invasive effect (this may maintain the infection and could be necessary for multiplication)
- Infectiousness
- Pathogenic mechanisms
- Infection dose to establish a disease
- Invasiveness
- Change in host specificity
- Ability to survive outside the host
- Mechanism for the transfer of genetic elements
- Biological stability
- Antibiotic resistance
- Toxicology
- Antiphagocytic factors
- Organotropisms
- Triggering of allergies
- Expression rate of the product (protein)
- Biological properties of the product.

Because this list does not claim to be complete, and particularly with respect to the administrative biosafety regulations, the reader is referred to the specific literature (Simon and Frommer, 1992) or the requirements according to, e.g., the EU Directives 90/219/
EEC ("contained use") and 90/220/EEC ("deliberate release") or the NIH Guidelines.

The "contained use" of microorganisms in classified facilities means that the technical measures taken are a lower or higher hurdle for the microorganisms to escape a given containment. Therefore, a 100% containment is both not possible and not necessary. Instead, the efficiency of containment must be both validated and appropriate for the microorganisms to be contained.

While most of the contained use applications deal with microorganisms which do not survive in the environment, the deliberate release of organisms into the environment with the purpose of their survival therein, at least for a certain time period, raises different questions. Evaluations have to consider the fact that possible adverse consequences as a result of the release of a naturally occurring exotic organism into a foreign and perhaps favorable environment (for review see OTA REPORT, 1993) is to be expected much greater than the release of an organism which has been modified by genetic engineering and which originates from a known, harmless organism with respect to the environment it is released into. This equally applies to plants, animals and microorganisms which may be harmful to the environment or not. Therefore, in regard to both the contained use and the deliberate release of organisms, the overall properties of the organism in question should be assessed when evaluating the risk and not the methods used to obtain the "modification".

A wide-ranging discussion of issues which arose in connection with the deliberate release of genetically modified organisms into the environment is not subject of this chapter, but is reviewed and well documented in the scientific literature (DOMSCH et al., 1987; TIEDJE et al., 1989) and is treated by MEDLEY and MCCAMMON in this volume.

4 Pathogenic Microorganisms

Microorganisms such as bacteria, viruses, parasites and fungi are available in large amounts (DAVIS et al., 1985) in soil, water and air, and only very few of them are pathogens. In fact, the great majority of microorganisms is harmless, and many are beneficial for animals as well as plants. Microorganisms pathogenic for man usually occur as a few types in species which mostly contain many non-pathogenic members (BERUFSGENNOSSENSCHAFT DER CHEMISCHEN INDUSTRIE, 1992) and can often be further divided into virulent and avirulent strains.

Since the end of the last century, the "Golden Era" of medical bacteriology, non-pathogenic and particularly highly pathogenic microorganisms have been investigated in basic research – safely for the researchers and workers concerned, the general population and the environment. With respect to industrial applications it must be stressed that particularly the fermentation of pathogenic bacteria, e.g., for the production of vaccines has proved to be very safe (KUENZI et al., 1985; TUINTENBURG, 1987).

Regarding legal aspects, it must be noted that international cooperation in the control of epidemics has had a legal basis since the beginning of the century (International Agreement on Sanitation, 21 June, 1926). Since the introduction of the Imperial Epidemics Law and, e.g., the German Epidemics Law of 1962, infectious diseases caused by more than 50 pathogenic microorganisms have to be reported and are therefore subject to administrative control. Furthermore, the German Epidemics Law controls not only the handling of sick patients or animals, but also rules that an authorization is required for the handling of pathogenic microorganisms, e.g., for research purposes, in clinical and biochemical laboratories. Similar regulations are in place in most OECD countries.

Important contributions to the understanding of biological processes stem from the analysis of the Darwinian process of genetic adaptation of organisms to various ecological niches. Such evolution occurs rapidly with mi-