February 24, 1975

TO: The Committee on Recombinant DNA Molecules
    Assembly of Life Sciences
    National Research Council
    National Academy of Sciences
    Washington, D.C., U.S.A.
    Paul Berg, Chairman


FROM: Working Party on potential biohazards associated
    with experimentation involving genetically altered
    microorganisms, with special reference to bacterial
    plasmids and phages

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Sirs:

We have the pleasure to transmit herewith for your consideration
proposed Guidelines on Potential Biohazards Associated with Experiments
Involving Genetically Altered Microorganisms. This report was written
in final form during two meetings, the first held in New York City,
November 7 - 10, 1974, the second in Palo Alto, California, February
20 - 23, 1975.

Respectfully,

PROPOSED GUIDELINES ON POTENTIAL BIOHAZARDS

ASSOCIATED WITH EXPERIMENTS INVOLVING

GENETICALLY ALTERED MICROORGANISMS

Prepared by:   Working Party on potential biohazards associated with
experimentation involving gentically altered microorganisms,
with special reference to bacterial plasmids and phages


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## Contents

## I. INTRODUCTION

### A. Scope and Purpose

1. <u>Scope</u>. These guidelines cover the modification of prokaryotic microorganisms by the introduction of foreign genetic information. Although this document has been prepared in response to a recommendation by the Committee on Recombinant DNA Molecules (Berg <u>et. al</u>., Proc. Nat. Acad. Sci., Wash. 71, 2593, 1974) that guidelines be devised for experiments involving "potentially hazardous recombinant DNA molecules", it is our view that there are certain other types of genetic manipulation and reconstruction that have so strong a logical kinship to the above that it would be artificial to omit them. At its broadest, then, this document will deal with <u>all</u> genetic manipulations involving the introduction into a prokaryotic species of genetic material that may or may not be native to that species and may be unlikely to be acquired by it in the natural environment.

For the purpose of this discussion, we will refer to a microorganism whose genome has been artificially modified by the addition of genetic information that is foreign to the species and unlikely to be acquired by it in nature as a <u>novel</u> <u>recombinant</u> <u>biotype</u> (or microorganisms). As current technology involves primarily the use of bacterial and phage genomes as carriers of foreign DNA, this term refers primarily to bacteria carrying foreign phages or plasmids or to native phages or plasmids that have had segments of foreign DNA added <u>in vitro</u>. While it includes, also, microorganisms with foreign DNA carried chromosomally, it excludes organisms produced from pre-existing ones by simple mutation.

The limitation of our recommendations to prokaryotic organisms is a practical one that is dictated by current limits of technology and of available information. These guidelines can and should be extended to eukaryotic microorganisms if and when those modifications along similar lines become feasible.

2. <u>Purpose</u>. The purpose of this document is two-fold: first to explore and detail the potential biohazards posed by a wide variety of classes of experiments involving recombinant microorganisms so as to raise the general level of awareness of these biohazards; and second, to make available suggestions for dealing with potential biohazards so that the individual need not rely entirely upon his or her own judgment.

Thus, it is hoped that the principle will be established that an open evaluation of biohazard potential and the adoption of an appropriate biohazard minimization procedure will be an integral part of experiments dealing with genetically altered microorganisms. Once this principle is accepted, a set of guidelines developed by an open, collective process that has taken into consideration the gamut of potentially conflicting interests will serve to enhance the safety and effectiveness of this line of research rather than to interfere with freedom of scientific inquiry, as has been feared.

### B. Background

Recent developments in DNA biochemistry and microbial genetics have made it possible to join <u>in vitro</u> segments of genetically active DNA from diverse sources, thus creating biologically active novel gene combinations that are exceedingly unlikely to occur naturally. Thus far, such recombinant <u>chimeras</u>

have involved the attachment of a DNA segment to a functional extrachromo-somal replicon of bacterial origin (a plasmid or a bacteriophage genome) and the introduction of the recombinant molecule into a suitable bacterial host cell where it replicates autonomously, serving to clone the added DNA segment. It is already certain that DNA from eukaryotic as well as from prokaryotic sources can thus be replicated and transcribed in bacterial hosts. Although it is not yet known whether or not eukaryotic DNA can be faithfully translated in bacteria, the consensus is that any barriers to translation could be by-passed by relatively straightforward manipulations.

This new technology thus constitutes a major breakthrough in molecular biology and gives rise to the possibility of important advances in at least four areas: (1) fundamental knowledge of gene structure, organization, and function; (2) genotypic modification of plants or animals to improve their usefulness to man (e.g., the development of nitrogen-fixing non-leguminous plants); (3) construction of bacteria or other such organisms able to produce rare and medically valuable biological substances such as insulin, growth hor-mone, etc.; and (4) genetic restitution of human hereditary diseases.

As with other major technological and scientific advances, gene grafting entails (along with its great potential benefits) at least the potential of serious and often unpredictable adverse consequences. Among these are bio-hazards that might result from the intentional or unintentional release into the environment of microorganisms carrying novel combinations of genes that have never existed before and are very unlikely to arise in the course of natural evolution. These biohazards would result, basically, from modifica-tion of the relationship between the organism and its environment - the gene-tically modified organism might be able to occupy new ecological niches or to function in a novel way within its normal environment, or both. One important subclass of these biohazards would involve an increase in the ability of a microorganism to cause human disease, including enhanced pathogenicity as well as increased resistance to eradication or treatment.

These possibilities have given rise to a significant level of concern among the general public as well as within the scientific community as there is ample precedent for the fear that the accidental introduction of organisms into new environments may have uncontrollable and sometimes dramatic untoward consequences. As examples of this, one might point to fire ants, killer bees, mudfish, snails, Xenopus toads and to Chestnut blight and Dutch elm disease. More germane, perhaps, to the present document is the serious biohazard inhe-rent in the astonishing spread in the space of a mere 30 years of bacterial plasmids carrying resistance to antibiotics consequent to the vast overuse and misuse of these valuable therapeutic agents.* The recent de novo appearance of such plasmids in Hemophilus influenzae and Streptococcus species suggests that their spread may by now have encompassed bacterial species to which they were never native before the present era.

The worry over possibilities such as these is not new; it has been ex-pressed through legislation to prevent the transportation of certain plant and

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* For documentation see, for example, the Report of the Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine (Chairman: Sir M. M. Swann) HMSO London, 1969

animal species between countries and between certain states in the U.S., and it has been expressed in the elaborate decontamination procedures to which leaving and re-entering space vehicles have been subjected. However, there has been little more than anguished hand-wringing over the antibiotic-induced spread of resistance plasmids. Perhaps the actions recommended in these pages to minimize the potential hazards of novel recombinant microorganisms will serve to stimulate similar actions to control the existant serious problem of antibiotic induced plasmid spread.

Concern over potential biohazards of novel microorganisms produced by in vitro genetic reconstruction was first articulated publicly in a report by a group of distinguished scientists, the Committee on Recombinant DNA molecules, published in the Proceedings of the National Acad. of Sci. U.S. (71:2593, 1974). in the summer of 1974. In this report, the Committee urged that a set of guidelines be developed to aid individual scientists to perform safely experiments involving the production and study of novel recombinant microorganisms. These guidelines would help in the assessment of the degree of danger involved and would recommend commensurate precautions. As a preliminary move, the Committee recommended a voluntary temporary deferral for two types of experiments and recommended that a third be performed with caution, until the appropriate guidelines were developed.

It appears that this deferral was largely successful and that the letter had the intended effect of setting in motion a number of independent inquiries to deal with the problem. One of these has already come to fruition in the form of a report, dated Dec. 13, 1974, to the British Parliament by a "working party on the experimental manipulation of the genetic composition of microorganisms" under the chairmanship of Lord Ashby. This report contains a very thoughtful analysis of the potential benefits and hazards attendant upon gene grafting research and outlines very briefly a set of broad recommendations.

The present document is in agreement with the philosophical position of the British report and is offered as a somewhat more detailed analysis of experimental systems intending to provide an explicit set of working guidelines for experimentation in this field. The two documents will thus be seen as complementary to one another, and their joint effect will be to replace the moratorium with specific recommendations as urged in the NAS Committee letter.

C. Principles

The philosophical position underlying this proposal and its contents is best expressed in the form of a set of basic principles, some of which are clearly established as facts, while others may be regarded as assumptions:

1. Since man has some measure of control over his actions, there is an operational dichotomy between the activities of man and the processes of the natural world. The distinction between "man-made" and "natural" is therefore meaningful and control of the former is both worthwhile and possible.

2. It is possible to modify profoundly the genome of a (micro) organism by artificial means involving the in vitro joining of unrelated DNA segments. Such modifications may find expression in the organism's phenotype as well as in its genetic constitution.

3. Modified (micro) organisms may behave in an unpredictable manner with

respect to the expression of foreign genes, and to the effect of this expression upon their ecological potential (including pathogenicity).

4. The genetic effects of these manipulations may be different from anything that ordinarily occurs during the natural process of evolution.

5. Historically unforeseen ecological effects of technological developments have been more often than not detrimental to man and his environment.

6. The release of a self-replicating entity into the environment will prove to be irreversible should that entity prove viable in the natural environment.

## D. Experimental systems and their safety

In view of the foregoing, a set of basic questions may be posed, which this proposal is a rather elaborate attempt to answer: Is it or is it not possible to evaluate a potential biohazard? i.e., How likely is it in any particular case that foreseeable or unforseeable adverse consequences will follow the release of a novel recombinant organism into the environment? Or, alternatively, granting the possibility of adverse consequences, how likely is it that a potentially hazardous but scientifically useful experimental system can be contained?

In general terms, the view to be developed here is that (a) it is often possible to evaluate to a greater or lesser extent (but rarely, if ever, fully) the potential biohazard associated with any novel biotype; (b) it is never possible to ensure absolute containment; but (c) it is often possible to reduce a potential biohazard to an acceptable level of risk without seriously compromising an experimental system.

Consequently, our recommendations will be based upon the following considerations:

(a) While it is not possible to ensure absolute containment, it is possible to develop containment procedures that are effective at various levels of stringency.

(b) Therefore, where it is judged that the escape of even a small number of experimental organisms would constitute a serious biohazard, the experiment should not be attempted.

(c) Where (b) is not the case, then containment procedures should be adopted whose stringency is based upon the best available evaluation of the biohazard potential as expressed as a permissible escape frequency for the novel recombinant organism – since escape frequency is really the only parameter involved in containment systems.

(d) Where possible, especially where evaluation of biohazard potential is difficult or impossible, the undesirable alternative of simply accepting the best available guess and acting accordingly should be circumvented by developing an experimental organism with very low potential for survival or transfer of its genetic material upon escape (see appendix C). Thus, a central consideration that will be dealt with here is the evaluation of normally used laboratory strains of bacteria with respect to their ecological potential and to the various possible ways of modifying them genetically so as to reduce their ecological potential and their ability to transfer DNA to other organisms.

(e) Finally, it must be stressed that while this set of guidelines is designed to help the investigator perform responsibly and with confidence those experiments deemed sufficiently important to justify whatever risk may be involved. These guidelines are not intended as a license to do unrestricted experimentation in this area. Experiments involving the construction of potentially hazardous novel recombinant biotypes should not be undertaken casually even within the containment framework appropriate for the level of risk involved.

## II. CLASSIFICATION OF EXPERIMENTS

### A. Considerations for the Assessment of Potential Biohazards

#### 1. Introduction

After deciding to construct a genetically altered microorganism, an investigator should consider each of the following points in deciding on an appropriate classification for the experiment to determine the type of containment necessary.

#### 2. Specific Considerations

##### a. Potential for Alteration of Pathogenicity.

For our purposes, pathogenicity and virulence are defined similarly as the "capacity to cause disease". How great is the known pathogenicity of the organisms involved? Will the genetic manipulation contemplated cause an increase in pathogenicity? If genetic information specifying traits that contribute to pathogenicity is used to construct a recombinant DNA molecule, then it is pertinent to ask:

i)   Is the ecology or reservoir of the virulence genes being changed?
ii)  Do these virulence genes occur naturally in the donor and recipient species in the general environment, in the local environment or in both?
iii) What is the potential for the transmission of these virulence genes from the modified organism to other microorganisms?

##### b. Potential for Dissemination.

If the genetically altered microorganism is pathogenic, can growth be controlled by antibiotics customarily used against the recipient strain? If antibiotic resistance is specified by the recombinant DNA, is this resistance to a drug of choice for treatment of infections by the microorganism? Is it a drug for which resistance is commonly expressed by the recipient organism? Is this drug resistance phenotype common locally among microorganisms of this type? Do the donor and recipient species naturally exchange genetic information? What is the potential for intercellular spread of the DNA chimera? When using plasmid DNA to construct recombinant molecules, do plasmids specify conjugal gene transfer? Are the recombinant DNA molecules normally restricted to an intracellular existence (as with plasmids) or do they normally persist extracellularly as encapsulated phage particles? Is the recipient lysogenic? Does the recipient possess plasmids (cryptic, conjugative or non-conjugative, autonomous or integrated)? Are the chimeric DNA molecules likely to recombine by natural means with other genetic material present in the recipient species? Is the recombinant DNA likely to undergo genetic alteration in its new host that may affect its biological potential?

##### c. Potential for Alteration of Ecology.

For our purposes, ecological potential is defined as the ability to occupy ecological habitats and the ability to alter the local ecosystem. Do the donor and recipient organisms share a common habitat? Does the donor organism possess phenotypic properties which, if expressed in the recipient, might substantially alter the ecological potential of the recipient? Will the genetically altered microorganism possess any unique metabolic properties that will alter the local ecosystem? Is it likely that the normal ecological habitat of the recipient will

be a factor affecting the biohazard potential when new metabolic capabilities are introduced?

d. Potential for Persistence in the Environment.

Would the recombinant molecules be expected to offer a biological advantage to the recipient organism which might affect its ecological potential? Does the genetically altered microorganism have a reduced susceptibility to disinfection or sterilization (e.g. resistance to ultraviolet irradiation, resistance to mercury-containing disinfectants, increased capacity for spore formation, etc.)?

e. Phenotypic Expression of Foreign Genes.

Are the phenotypic traits specified by the foreign DNA known to be expressed by strains of the recipient species? What is the likelihood of accurate transcription, translation and phenotypic expression of the foreign DNA in the recipient? What biological consequences are likely to result from their phenotypic expression in the recipient?

f. Availability of Genetic Information About Organisms Involved.

How well characterized are the organisms? Have they been isolated recently or are they well-studied laboratory strains?

g. Purity and Characterization of DNA Used in Forming Recombinant Molecules.

Are the DNA molecules used in the experiment derived from plasmid or phage species having well-characterized genetic and molecular properties? Does the DNA sample represent a single molecular species or does it contain a random assortment of molecules or fragments?

3. General Considerations

a. When an investigator is in doubt, the experiment should be placed in the higher of two classes being considered.

b. Since there is a corresponding increase in potential biohazard when large numbers of microorganisms are used, investigators should classify large-scale experiments as more hazardous than those in which the new microorganism was initially constructed which involved relatively small numbers of cells.

c. It should be recognized that mutagenesis may alter the host range of bacteriophages and plasmids used as cloning vehicles. It is therefore prudent following recent mutagenesis of either genetically altered microorganisms or cloning vehicles to place the experiment in the next higher containment class until it has been determined that the host range has been unaltered.

B. Classes of Experiments

Experiments on the construction of genetically altered microorganisms have been categorized into six classes in terms of severity of the known or potential biohazards as follows:

1. Class I Experiment: Class I includes experiments in which the biohazard can be assessed and is known to be insignificant. More specifically, all of the following conditions must be fulfilled:

a.  The pathogenicity of the donor and recipient organisms is
    minimal and is known to be unchanged by the procedure in
    question, and
b.  It is known that dissemination of the organisms involved
    is fully and easily controllable, and
c.  All DNA species involved are well characterized and their
    genetic properties are well understood, and
d.  The experiment does not alter the ecological potential of
    the recipient compared to other strains of the same species,
    and
e.  The genotypic and phenotypic properties under study occur
    naturally in the recipient species or can be readily trans-
    mitted to strains of the recipient species.

Examples of Class I Experiment:  Gene transfer or genetic recombination
between laboratory strains of Escherichia coli such as K-12, B, C and 15.
This includes conjugal transfer by F$^+$, F'-containing and Hfr donors.  See
Appendix B for additional examples.

2.  Class II experiment:  Class II includes experiments in which the biohazards
can be reasonably assessed and from what is known about them one can expect them
to be minimal.  More specifically, all of the following conditions must be ful-
filled:

a.  The species used to construct the genetically altered micro-
    organism have either low or moderate pathogenicity similar to
    that expressed by Salmonella typhimurium, Staphylococcus aureus
    or Haemophilus influenzae, and
b.  The genetic material used to construct the altered microorganism
    is derived from organisms known to be capable of transmitting
    genetic information to the recipient, and
c.  The genetically altered microorganism should not have ecological
    potentials greater than can be conferred as a consequence of
    normally occurring genetic exchange processes, and
d.  The genetically altered microorganism does not contain genetic
    information that would prevent effective treatment of infections
    caused by it.

It should be noted that in some instances an organism serving as a DNA donor
may have a greater potential either to exhibit pathogenicity or to occupy unique
ecological habitats than the recipient organisms and hence poses a greater poten-
tial biohazard than the recipient.  In this event it is the potential biohazards
associated with the donor of the DNA that determines the classification of the
experiment.

Examples of Class II Experiment:  The construction of recombinant molecules
either in vitro or in vivo between R and F' plasmids, between Col and F' plasmids,
between Col and F' plasmids or between bacteriophage λ and a Col or R plasmid
when introduced into E. coli.  See Appendix B for additional examples.

Classes III, IV and V Experiments include:

(i)  all constructions of genetically altered microorganisms which use donor and
recipient organisms that ordinarily do not exchange genetic information and

(ii) some constructions of genetically altered microorganisms which use organisms which ordinarily do exchange genetic information.

3. Class III Experiment: Class III includes experiments in which the biohazards usually cannot be totally predicted. However, on the basis of all available information, it is considered likely that:

   a. The recombinant DNA will not contribute significantly increased pathogenicity to the recipient, nor significantly alter its ecological potential, and

   b. Pathogenicity of the genetically altered microorganism or its parents is minimal (e.g., B. subtilis), low (e.g., E. coli) or moderate (e.g., S. typhimurium), but not severe (e.g., Y. pestis), and

   c. The genetically altered microorganism does not contain information that would prevent effective treatment of infections caused by it.

Examples of Class III Experiment: Construction of a hybrid plasmid or phage that includes an antibiotic resistance gene derived from S. aureus when introduced into E. coli, so long as genes conferring resistance to that antibiotic are found in E. coli. Construction of a hybrid plasmid or phage that includes ribosomal genes from Xenopus laevis or random fragments of Drosophila melangaster DNA when introduced into E. coli. See Appendix B for additional examples.

4. Class IV Experiment: Class IV, like Class III, includes experiments in which the biohazards are usually unknown, and cannot be accurately assessed, but because of the known genotypic and/or phenotypic properties of the DNA and/or organisms used to construct the genetically altered microorganism, they are judged to be potentially significant in affecting either the ecologic potential or pathogenicity of the recipient organism.

Examples of Class IV Experiment: Construction of a hybrid between random DNA fragments from S. pyogenes and an F'lac plasmid and its introduction into E. coli. Construction of hybrids between random DNA fragments from normal human fibroblasts and an E. coli plasmid or phage when introduced into E. coli. Construction of a hybrid between either λ or plasmid DNA and the genes specifying synthesis of cellulase and/or ligninase from Polyporus annosus and its introduction into E. coli. See Appendix B for additional examples.

5. Class V Experiment: Class V also includes experiments in which the biohazards are usually unknown, but because of the known genotypic and/or phenotypic properties of the DNA and/or the organisms used in the construction of the genetically altered microorganism, they are judged to be severe in affecting either the ecological potential or pathogenicity of the recipient organism.

Examples of Class V Experiment: The construction of a recombinant DNA molecule between the plasmid from S. aureus determining exfoliative toxin and an R plasmid or λ and its introduction into E. coli. Construction of hybrids between E. coli phage or plasmid DNA, and unknown genes from Y. pestis, B. anthracis, or B. abortus, when the hybrid is introduced into E. coli. See Appendix B for additional examples.

6. Class VI Experiment: Class VI includes experiments in which the biohazards are judged to be of such great potential severity as to preclude performance of the experiment at the present time under any circumstances, and regardless of containment conditions.

Example of Class VI Experiment: The introduction by any means of the genes for botulinum toxin biosynthesis into E. coli. See Appendix B for additional examples.

## C. Summary of Classification

We have categorized experiments involving the construction of genetically altered microorganisms into six classes. The assignment of experiments to Classes I and II involves little difficulty, since genetic exchange between the organisms used occurs normally. Classes III, IV and V experiments, however, primarily include the construction of genetically altered microorganisms which use donor and recipients that ordinarily do not exchange genetic material. We recognize that in many specific instances the distinction between a Class III and a Class IV or between a Class IV and Class V experiment will be difficult to make since these classes include experiments in which the potential biohazards cannot be totally assessed. Ultimately, the distinction will depend upon the ecology of the recipient bacterial species, the nature of the cloning vehicle and the likelihood of phenotypic changes resulting from introduction of the recombinant DNA.

A natural tendency is to consider changes in pathogenicity as the primary biohazard concern since these come to mind most readily when considering microorganisms; other changes which may affect the fundamental ecological potential, adaptability, metabolism, etc. of a recipient organism may be more subtle and much more difficult to assess than pathogenicity. However, these alterations may potentially present an equal or greater biohazard. We can offer only a relatively few guidelines to help an investigator in determining the class assignment of an experiment in Classes III, IV or V; perhaps the most critical is the extent of characterization of the genetic material being employed in the experiment since we believe that the potential biohazards of a purified and well-characterized donor DNA species are more easily assessed than the biohazards inherent in the introduction of a random assortment of DNA fragments.

## III. CONTAINMENT PRINCIPLES AND PROCEDURES

### A. Introduction and General Recommendations

Biological safety and environmental control programs for dealing with pathogenic bacteria have been implemented in clinical and biomedical research laboratories for many years (refs. 1-12). Once a potential biohazard has been defined and the risk has been assessed, the major thrust of the procedures employed to minimize the biohazard involves steps to limit risk to the laboratory worker and to prevent the escape of potentially hazardous biological material.

Many of the basic problems of containment that face an investigator studying recombinant DNA in a microbial species are similar to those faced in every medical microbiology laboratory. A clinical specimen received for microbiological analysis may contain an etiologic agent ranging from those of ordinary potential hazard to those which may require the most stringent conditions for their containment. One cannot be certain until the etiologic agent is isolated and its known pathogenicity (i.e. its potential hazard) assessed. By the same token, an investigator who employs a random assortment of DNA molecules for construction of recombinant DNA molecules could, at least in theory, isolate a variety of novel transformant bacterial clones which range in their potential biohazard. The following safety considerations are applicable to all procedures involving etiological agents in the clinical laboratory. As such they may be considered as prudent standard procedures for those working with bacteria containing recombinant DNA molecules. Obviously, those investigators working with animal or plant viruses will need to satisfy the special containment problems inherent in the laboratory manipulation of these agents.

The procedures listed below are a reiteration of long-standing microbiological practices and simply reenforce the concept that microbiological safety is a matter of good working habits. All of the general recommendations listed below are desirable for all classes of experiments, although we recognize that they are not specifically needed for the safe handling or containment of all agents.

1.  Consequently, our primary recommendation for containment of potential biohazards is that all individuals planning research with recombinant DNA molecules in bacteria receive adequate training in microbiology. Such training should not be construed to mean that one needs to learn only aseptic techniques or the procedures for handling potentially infectious material. Rather, investigators cannot afford to ignore the basic biology of the microorganism -- its ecology, innate pathogenicity, physiology, growth requirements, etc. In short, an investigator must try to think in microbiological terms before initiating experiments that could potentially affect the basic ecology and/or pathogenic potential of an organism that serves as a carrier for a recombinant DNA molecule. The microorganism is not simply a "warm body" to house a recombinant DNA molecule of interest.

It is axiomatic that no safety facilities or equipment (no matter now sophisticated) can take the place of an investigator's responsibility. In terms of biological safety, the principal investigator cannot delegate, reassign, abandon or ignore his or her responsibility that adequate safety training be given to all laboratory personnel. We have appended a list of books and other publications which deal with the general topics of laboratory safety, biohazards in biological research and the handling of specific bacterial agents which may prove useful as a source of specific information.

2.  As a general principle, doors to laboratories in which potentially biohazardous material is handled should be kept closed.

3.  Eating, drinking or smoking in the laboratory is undesirable and in areas in which potentially biohazardous material is handled should be specifically forbidden.

4.   The most frequent causes of laboratory acquired infections are accidental oral aspiration of infectious material through a pipette, accidental inocula-tion with syringe needles and animal bites (10,11).  A further important cause of both laboratory acquired infections and contamination of the environment is aerosols from centrifugation, blending, loose needles on syringes and even the improper flame sterilization of contaminated inoculating loops and needles. (see chapters by Dimmick et.al., ref. 1) As minimal recommendations, handwashing by laboratory personnel should be encouraged and direct mouth pipetting should be discouraged.  The use of cotton plugged pipettes may be acceptable for agents of low or moderate hazard but a mechanical pipetting device is preferable.  Special aerosol precautions are generally not required for most bacterial species, but their use deserves careful consideration.

5.   Bacterial cultures and potentially hazardous DNA should be disinfected or sterilized by autoclaving.  The laboratory should be cleaned, work surfaces de-contaminated and all contaminated material placed in discard pans (preferably covered) containing a suitable disinfectant or autoclaved at the end of the day. The use of specific disinfectants cannot be recommended here, since they will vary from bacterial species to bacterial species and, additionally, must be capable of rendering nucleic acid solutions "non-infectious".  One should not accept manufac-turer's claims for disinfectant effectiveness -- there is no substitute for a use-test evaluation performed against the microorganism and nucleic acid solutions pro-cessed in the laboratory.

6.   Any research group working with agents with a known or potential biohazard should have an emergency plan, including a clean-up procedure to follow if an accident contaminates personnel or environment.  Here again, the principal inves-tigator must insure that everyone in the laboratory is familiar with both the potential hazards of the work and the emergency plan.

7.   If a research group is working with a known bacterial pathogen for which a vaccine is available, all workers should be vaccinated.  Immunization is not, how-ever, a license for procedural short-cuts nor a substitute for safe laboratory practice.


B.   Levels of Containment

     The containment procedures proposed are designed to match the previously defined classes of experiments involving novel recombinant bacteria.

     Since containment cannot be absolute, the rationale underlying these contain-ment recommendations is that the greater the potential biohazard, the more stringent should be the containment.  In our judgment, each level of containment implies an acceptable level of protection for laboratory workers and an acceptably low proba-bility of escape for the organisms involved.

Class I Experiments:  Requires no special containment other than practice of stan-dard aseptic technique (i.e. use of procedures to maintain pure cultures and dis-infection of discarded materials).

Class II Experiments:  The basic criteria for this category are those minimal operating procedures employed in a clinical microbiology laboratory.  These are:

     1.   Eating, drinking and smoking are forbidden in the laboratory.
     2.   Laboratory coats are required during handling of biohazardous
          material.  These should not be worn outside the work area.

3. Cotton-plugged pipettes or mechanical pipetting devices are required. The latter are preferable.
4. Routine disinfection of work surfaces and prompt disinfection or sterilization of all contaminated material should be carried out.
5. Immunization of personnel is required for experimenting with S. typhi, V. cholerae, C. diphtheriae and C. tetani.
6. Specific aerosol precautions are required (see below, III, 3) when large volumes (6 or more liters) of biohazardous materials are centrifuged.

Class III Experiments: The same minimal standards described for Class II are applicable with the added provisions that:

1. No mouth pipetting of potentially biohazardous material is permitted. Mechanical pipetting devices are required.
2. The experiments are performed in laboratories that are under controlled access. This does not require a separate room in which no other work is concurrently being conducted. Rather, the intent of this containment feature is to exclude extraneous persons from the area and, hence, reduce the number of exposed individuals should a laboratory spill or other accident occur. Appropriate biohazard signs will be posted on the doors of laboratories during biohazardous experimentation as well as on the doors of storage areas or cabinets containing potentially hazardous materials. Visitors to these work areas are prohibited unless they have permission from the investigator in charge who is responsible for the visitors while they are in the area.
3. Specific aerosol precautions are mandatory (see for example, R.L. Dimmick, W.F. Voge and M.A. Chatigny. Potential for accidental Microbial Aerosol Transmission in the Biological Laboratory In Biohazards in Biological Research ed A. Hillman, M.N. Oxman and R. Pollack. Cold Spring Harbor Laboratory, 1973, pp. 246-266). Thus, syringes to which the needle is firmly fixed (e.g. Luer-Lok) should be used. Screw-capped safety cups on centrifuge tubes are required when centrifuging biohazardous materials. Operation of centrifuges in hoods or other enclosed areas is desirable. Safety equipment to prevent the dissemination of aerosols generated by blending, sonication, centrifugation, etc. is commercially available (1).

Class IV Experiments: The same minimal standards required for Class III experiments are applicable with the added provisions that:

1. At the minimum, a partial containment cabinet (see W.E. Barkly, ref. 1) or its equivalent should be used for experiments in this category. This is a local exhaust ventilation hood with a limited front opening in which air entering through is subjected to high efficiency particulate air (Hepa) filtration or incinerated before being exhausted from the area.
2. Special aerosol precautions are mandatory for experiments in this class. Centrifuges, blenders and other equipment capable of creating aerosols should be operated in separate isolation rooms or hoods (see Dimmick, et.al. and Bonn, ref. 1). The standard biological hazard sign used for highly infectious agents (op. cit. p. I-22) will be posted on cabinets, freezers, refrigerators, and/ or work area where biohazardous materials are kept or are being used. Only personnel who work in the laboratory may enter the

area when this sign is posted.

Class V Experiments:  The potential severity of  risk entailed in Class V experiments dictates that they be carried out in specially constructed facilities used to contain highly infectious microbiological agents.  In such facilities, personnel enter through a change room, shower, put on special protective clothing (i.e. disposable gloves, gowns, and foot covers), walk through a disinfecting foot bath, and enter an enclosed laboratory area that contains an "absolute containment cabinet" (Class III see Barkly, op. cit.).  These cabinets are provided with ultra-filters that can be sterilized, and the hood must be capable of  being fumigated. The room in which the hood is located should be completely sealed, with vapor locks around the door, light fixtures sealed into the ceiling, and all air coming out of the room must pass through appropriate Hepa filters.  A double door autoclave should be mounted in the wall of the room so that after sterilization, materials can be taken out of the autoclave into another room.  Upon completion of experimental procedures, the personnel must walk through a foot bath, dispose of clothing which would be sterilized, shower, go back into an entrance room, and put on their normal street clothes.


Class VI Experiments:  No acceptable level of containment compatible with potential biohazard.

C.   Pertinent References

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IV.   RECOMMENDATIONS FOR THE IMPLEMENTATION OF GUIDELINES

A.  Committees specifically dealing with potential biohazards associated with recombinant DNA should be established at every academic institution and commercial organization where experiments with potential or known biohazards are proposed.

B.  All investigators wishing to carry out experiments involving possible biohazards would be required to submit a proposal to the institutional committee, indicating the purpose of the experiment, the explicit benefits to be derived, and an assessment of the potential biohazards and precautions for containment that are proposed.

C.  The responsibilities of the committee would be to familiarize themselves with the extent of potential biohazards and the necessary measures for their minimization and containment.  It should ensure that no experiments of this nature are carried out unless the investigator had submitted such a proposal. It would ensure that the investigator was familiar with appropriate guidelines and that a thorough review and assessment of the biohazards and their containment had been carried out.  It would then evaluate the proposal and any supporting evidence and would make its recommendation on the proposed research.

D.  The submitted proposal and the committee's review would be filed as public documents in a biohazards repository at the institution.  This file would be submitted with all grant proposals and applications related to the experiments. Any subsequent modifications to the research program which materially affected the extent of the biohazards would require a new proposal and a further review. Progress reports would be required at yearly intervals to ensure that the proposed experiments, precautions and containment were adhered to.  A complete file of all approval programs under study would also be kept in a federal repository and would be available for publication.  The file of documents on each proposal would be made available by the investigator to those journals where publication policy required appropriate documentation.

E.  Since the types of experiments under discussion usually require only minimal equipment, of the type generally available at most academic institutions, it is recognized that in the absence of continual supervision or monitoring, the responsibility to pursue such a program of research rests finally with the investigator.  We believe therefore that the Principal Investigator must shoulder the ultimate responsibility for the experiments.  Thus, responsibility for proceeding with an experiment should not be shifted from the PI to a local committee, absolving the PI from responsibilities.  The local committee should make recommendations and provide advice but cannot approve a program.  Thus, even in face of a favorable review by the committee, an investigator would need to ensure that a program satisfied the requirements of the guidelines.  In the event that the investigator decided to ignore recommendations of the local committee, supporting evidence for proceeding with the experiments should be obtained from outside the institution which would justify the ultimate course of action.

[We believe that a combination of scientific integrity combined with peer pressure generated in the face of public availability and scrutiny of the documents mentioned above, will result in strict adherence to the guidelines, while at the same time avoiding the extremes of the approval of hazardous series of experiments by a poorly informed local committee or a veto by a local committee of experiments which would be generally accepted as valid and worthwhile under the conditions of containment that have been proposed.]

F.   No potentially ecologically hazardous microorganism would be released into the environment intentionally without the approval of an international body which would be duly constituted to make judgements on such release.

G.   All individuals embarking upon experiments categorized as Class II to V, should receive training in the handling of potential or infectious material and must be familiar with the NIH and ASM guidelines (See refs 2 and 3; also 9, 10 and 11) of experimental use of such materials.

[An experimenter who has been well trained in working with pathogenic micro-organisms and who is familiar with the ASM Handbook of Clinical Microbiology Guidelines should have sufficient expertise to be able to make appropriate judgements regarding the classification of individual experiments in the laboratory situation.  Familiarity with this information should enable him to prescribe appropriate containment procedures for that particular type of experiment and will also enable him to make correct judgement about the type of training required for technical personnel that may participate in the experiment.]

H.   In those countries where experiments of the type referred to in this proposal are being carried out, it would seem necessary that national bodies sould be constituted to establish, monitor and promulgate guidelines.  An international body should also be established

1.   to consult with and advise national organizations on the development and implementation of guidelines;
2.   to encourage the maintainance of uniform standards through-out the world;
3.   to coordinate and periodically review the efficiency and applicability of international guidelines; and
4.   to authorize any dissemination into the environment of new recombinant types that are likely to produce signifi-cant ecological effects.

## V. CONCLUSIONS

We believe that considerable benefits are likely to result from experiments involving the genetic alteration of microorganisms. The range of possible benefits extends from the use of these techniques to add to our knowledge of basic biological phenomena, to possible practical applications in the areas of agriculture and medicine.

We believe also that a scale of risks exists in the construction of genetically altered microorganisms, and we are uncomfortable about our inability to assess precisely the extent of such risks for many types of experiments. However, we believe that the containment procedures described in this proposal will reduce any risk to laboratory workers and to the environment to a level that is acceptably low and which will allow investigators to carry out research in this area. We believe that certain experiments should presently not be carried out under any circumstances (i.e. Class VI), but that most experiments can be done if containment facilities appropriate to the risk are utilized.

We recommend that specific steps be taken as soon as possible to develop cloning vehicle-host systems which will further reduce biohazard potential, will minimize the necessity of elaborate containment facilities, and will obviate judgements which must necessarily be based on little or no data at the present time. Specifically, we recommend that special sponsored programs be instituted immediately for the development and testing of such systems. We recommend also the prompt establishment of experimental programs intended to evaluate more fully the potential hazards that may be involved in the genetic alteration of microorganisms.

We believe that perhaps the greatest potential for biohazards involving genetic alteration of microorganisms relates to possible military applications. We believe strongly that construction of genetically altered microorganisms for any military purpose should be expressly prohibited by international treaty, and we urge that such prohibition be agreed upon as expeditiously as possible.

Other recommendations for implementation of the guidelines proposed in this report are contained in Section IV.

# THE ECOLOGY OF PLASMIDS AND BACTERIOPHAGES

## A. Plasmid Ecology

The term R plasmid came into use in 1959 with the discovery in Japan that multiple antibiotic resistance could be transmitted by conjugation from strains of resistant epidemic Shigella to drug sensitive Escherichia coli. The significance of this observation became clear over the next four years when it became apparent that the problem of transmissible antibiotic resistance was a worldwide problem with broad implications to medicine. It is now quite clear that when a clinical isolate of an enteric organism displays multiple antibiotic resistance, the resistance is most often mediated by an R plasmid. Moreover, recent surveys of the resident flora of asymptomatic individuals in the community-at-large and the bacteria in the community environment have shown that the incidence of drug resistance has been increasing at a remarkable rate. The increase in the incidence of R plasmids in human populations is, of course, directly linked to the use of antimicrobial agents in medicine. Domestic animals also have shown a parallel emergence of resistant strains. However, the entire problem of bacterial drug resistance in animals is complicated by the fact that most classes of animals grown for food are fed diets containing antibiotic supplements for the stimulation of growth. There has, therefore, been an enormous selection for microorganisms containing plasmids because of a massive alteration in the environment.

The microbial geneticist was attracted to the study of R plasmids not only from the standpoint of their similarity to the classical F transfer system, but also from the standpoint of public health, and the        unique opportunity to monitor the extent of change and the genetic basis of change in natural bacterial populations. The increased attention to natural bacterial populations has led to a broad view of the ecology of bacterial plasmids. For example, fully one-third of Escherichia coli from asymptomatic human and domestic animal populations possess at least one self-transmissible (conjugative) plasmid that confers few or no known phenotypic traits other than conjugal fertility. Bacterial plasmids confer a far greater diversity of phenotypic traits upon the bacteria that possess them than 'simply' antibiotic resistance or genes (such as enterotoxin biosynthesis) that contribute to bacterial pathogenicity. Plasmids have been identified in a variety of bacterial genera and associated with such diverse functions as the control of lactose fermentation in Streptococcus lactis, sporulation in Bacillus pumilus, and camphor degradation in species of Pseudomonas. There has been a growing appreciation of the fact that the genes for antibiotic resistance, toxin biosynthesis and other genes such as lactose fermentation, which are of 'transient' evolutionary advantage may be carried by virtually identical molecular vehicles. Thus, it is possible to isolate conjugative plasmids which are identical in over 80 percent of their molecular lengths but which carry on the one hand antibiotic resistance genes, on the other hand genes for the biosynthesis of enterotoxin and, in yet another instance, genes which control the utilization of lactose; there are numerous other examples to suggest that the same plasmid wearing different phenotypic garb is often isolated independently in several laboratories. To a great extent it appears that the genetic information which controls essential plasmid functions such as replication, the distribution of progeny replicas and, to a somewhat lesser degree, transfer functions is conserved; indeed, plasmids, regardless of phenotype, can be 'speciated' by genetic and molecular studies.

However, the origin of and relationships among plasmid genes which determine antibiotic resistance and other properties is the subject of considerable speculation. In some instances, for example certain types of ampicillin and tetracycline resistance, it now appears that the genes in question reside upon a segment of DNA which is readily translocated from replicon to replicon and that this event can occur in recombination deficient (rec⁻) bacteria. More recent studies have shown that this 'rec' independent translocation of genes is not restricted to antibiotic resistance determinants but affects other plasmid and host genes as well.

B.  Plasmid Transmission in vivo

1.  Introduction

Given a conjugative R plasmid residing within an enteric organism inha-
biting the bowel, the immediate thought might be that R plasmid transfer could
occur quite readily to other enterobacterial strains.  This conclusion would
seem justified by the laboratory observation that R plasmid transfer from one
strain to another can usually be demonstrated without difficulty even when it
occurs at a low rate.  The bowel of an animal is a far cry from a test tube,
however, and from a practical point of view it is important to ask to what
extent transfer occurs in vivo.  The available evidence indicates that trans-
fer does not occur on so grand a scale in vivo as it does in vitro.  The
following sections which deal specifically with the strain E. coli K-12 outline
the general parameters of in vivo genetic transmission and will, hopefully,
help interested investigators to evaluate the steps that must be taken to pre-
vent the dissemination of recombinant plasmid DNA.

2.  The "Infectivity of E. coli K-12"

Thus far, the 'cloning' of recombinant DNA molecules has been restricted
to substrains of Escherichia coli K-12, B or genetic hybrids of the two.  Both
E. coli K-12 and B are long established laboratory strains which were initially
isolated from man.  One of the first questions to be asked, therefore, is how
commonly these E. coli substrains can colonize the human or animal intestine.
Although this precise question has not been studied extensively, it has been
shown that E. coli K-12 is a very poor colonizer of the normal bowel.  For
example, after feeding of between $5 \times 10^{11}$ to $1 \times 10^{12}$ E. coli K-12 cells to
calves, only about $10^7$ cells can be recovered per gram of feces in 24 hours and
by 72 hours cannot be identified at all ($< 10$ cells) (3).  Similarly in man,
ingestion of $10^9$ cells does not normally lead to colonization, indeed, the detec-
tion of more than 100 K-12 cells/gm after 24 hours is rare.  Consequently, it
appears that E. coli K-12 has very little inherent capacity to colonize man.

There are, however, exceptions to this general rule.  If the normal flora
of man or animals is disrupted, for example, by therapeutic levels of anti-
biotics, the ingestion of E. coli K-12 bearing the resistance determinants to
these antibiotics leads to colonization at easily detectable levels (about $10^5$
per gm of feces).  Similarly, individuals who have had surgical treatment for
stomach or bowel disorders are far more easily colonized by all enteric species
(including E. coli K-12).  Finally any substance which 'protects' an ingested
organism from the acidity of the stomach leads often to a higher level of K-12
excretion (although subsequent colonization of the normal bowel does not occur,
the length of time of excretion may be increased by a few days).  Therefore, a
few simple rules appear to be prudent with regard to handling E. coli K-12, par-
ticularly when they contain either recombinant DNA molecules or naturally
occurring plasmids for that matter:

   a.  The usual laboratory procedures employed in dealing with enteric
       pathogens should be followed as described above.
   b.  Individuals who are receiving antibiotic therapy should not work
       with the strains during the period they are receiving therapy and
       for seven days after the cessation of therapy.

> c. Individuals who have functional intestinal disorders and those who have had surgical removal of part of the stomach or bowel should not work with these strains.
>
> d. Individuals who take large amounts of antacids should be aware that they are more readily colonized by ingested bacteria. Obviously, the usual laboratory precaution of no eating in the laboratory should be followed.

3. Gene Transfer in the Gut

Although E. coli K-12 and B derivatives do not usually actively multiply and colonize the normal animal bowel, the organisms that survive the acidity of the stomach and other natural host defenses, remain viable and can act as genetic recipients or genetic donors under the proper circumstances. In so far as we are aware all of the recombinant DNA molecules that have been prepared thus far are nonconjugative, that is, they do not inherently have the ability to initiate transfer of DNA. Nonetheless, these nonconjugative plasmids can be mobilized by a transfer plasmid (such as the classical F plasmid) residing in the same cell. A possible scenario for extension of the reservoir of a recombinant DNA molecule could be as follows: A research worker ingests E. coli K-12 containing a recombinant DNA plasmid. The surviving cells while in the gut, engaged in conjugation with a member of the normal flora containing a transfer plasmid. (Note: about 38% of all E. coli strains from asymptomatic animals and man harbor at least one transfer plasmid.) The converted K-12 organism containing both the transfer plasmid and the recombinant plasmid mates with a member of the normal gut flora and the recombinant plasmid is transferred. The latter strain is fully capable of survival in the gut and can, in turn, mate with other strains.

This hypothetical sequence of events has a certain probability that can be calculated on the basis of laboratory experiments at 1 in $10^{-6}$ – 1 in $10^{-8}$ per bacterial cell. Experiments of this nature suggest, however, that the probability of this occurrence in the normal gut is on the order of 1 in $10^{-12}$ to 1 x $10^{-14}$. This differential between laboratory and gut illustrates the concept that the best defense against R plasmid and other gene transfer is a normal gut and gut flora. Conditions in the bowel such as Eh, pH, fatty acid concentration, etc. are simply not optimal for genetic transfer. Indeed these same physiological conditions of the normal bowel provide us with one of the major natural defense mechanisms against infection by enteric pathogens. A major exception is, again, instances in which the normal flora has been modified by antibiotic treatment or if there is a functional or pathological bowel disorder. Under these circumstances, the probability of _in vivo_ transfer increases to an average of 1 x $10^{-6}$ to 1 x $10^{-9}$. Thus, the parameters which affect the colonization of E. coli K-12 likewise affect the probability of genetic transmission and the guidelines listed above apply to the prevention of _in vivo_ genetic transmission. Of course, the probability of gene transfer by an ingested K-12 is exceedingly low particularly after the first 24 hours of ingestion. In our judgement, gene transfer from E. coli K-12 is not a significant hazard so long as normal precautions of the bacteriology laboratory and the containment guidelines listed earlier are followed.

4. Gene Transfer Outside the Gut

There is one situation in which gene transfer might contribute to the dissemination of recombinant plasmid species. This situation could result from an

unfortunately common practice in some laboratories, namely the discarding of culture supernatants and even viable cultures of E. coli K-12 and other "non-pathogenic" bacterial species into the laboratory sink which empties into the community sewer system.  On the face of the matter it might be imagined that virtually any form of sewage treatment would effectively destroy the bacteria. This assumption is totally unfounded, however.  For example, in Washington, D.C., during periods of heavy water use or during a period of heavy rainfall, it is quite possible that a high proportion of organisms disposed of down a drain would reach the Potomac River where E. coli counts in excess of $10^7/100$ ml are not uncommon.  (Note that this situation is found, of course, in most urban areas).  There is relatively little data available concerning the frequency of genetic exchange in water.  However, it is known that fecal E. coli harboring R plasmids have a very good survival potential in sewage and in river water. At any rate, it should be reemphasized that it is not a good practice to dispose of any viable bacterial culture into the community sewage disposal system. This is, of course, particularly critical with respect to cultures containing recombinant plasmid species or naturally occurring R plasmids for that matter. All such strains should be considered to have at least some minimal degree of hazard and treated with the common sense experimental practices detailed in the section on containment.  Similarly, one does not know the potential hazards of gene transfer on bench tops, etc. which may be contaminated by spills. Again, one needs to reemphasize the basic methodology that is taught to every beginning student of microbiology.

Roughly 10-15% of normal, asymptomatic individuals harbor E. coli and other coloiform organisms in their nasopharynx.  It is not known with any degree of certainty to what extent well-established laboratory strains of E. coli such as K-12 may colonize this anatomical region.  This possibility should be investigated.

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## C.  Bacteriophage Ecology

The literature on bacteriophage is enormous and it would be obviously futile to attempt to summarize all that is known about their distribution in nature.  Virulent bacteriophages are capable of only a productive life cycle in bacteria so that their propagation invariably leads to death and lysis of the bacterial host.  Temperate phages on the other hand, as exemplified by the phage $\lambda$ of E. coli K-12, lead a sort of Jekyll-Hyde existence in bacteria. They are capable of productive growth (lysis) or may become inserted into the bacterial chromosome and so assume a relatively passive role (lysogeny).  The decision to lyse or insert is under the control of a complex system of genetically controlled biochemical 'switches' and it is possible for the inserted bacteriophage chromosome (called a prophage) to become induced to a productive

growth cycle after peacefully coexisting with the bacterial host for many generations. Other temperate phages such as P1, have prophages that do not integrate into the bacterial chromosome but rather replicate while attached to the bacterial inner cell membrane. As such, these prophages are plasmids.

One need only examine filtrates of fecal suspensions, raw sewage, soil, water, unpasteurized diary products or even diseased tissue to learn that both virulent and temperate phages are very common in nature. The systematic search of bacterial species for the presence of a carried temperate phage is so often successful that some writers have been moved to remark that it is difficult to believe that there are many bacterial cells that are not carrying at least one temperate phage! This certainly seems to be the case, for example, when speaking of staphylococci but for other bacterial species the reported incidence of carried phage varies from 2% to 94%. Since for the major purpose of this document we are primarily interested in the strain E. coli K-12 and the bacteriophage λ and its derivatives, it is probably best to simply focus on how often E. coli species of natural origin carry phages which can also infect E. coli K-12 and how many of these phages are 'lambdoid'.

Apparently phages resembling λ are not uncommon in wild-type E. coli. For example some 20 years ago Jacob and Wollman found that 32 or 500 fecal E. coli carried temperate phages capable of propagation on an E. coli K-12 derivative. Among these 32 phages, 3 were apparently identical to λ and at least six others could recombine with λ. All of the other phages could be effectively carried by E. coli K-12 but were not related to λ. More recent unpublished observations from several laboratories have confirmed these findings and it is probably fair to say that some 8% to 10% of all fecal E. coli harbor at least one phage capable of infecting E. coli K-12 and that from 1% to 2% of fecal E. coli carry a phage that is closely related to λ.

Some temperate phages alter profoundly the properties of bacteria that become lysogenized. This process has been termed phage conversion and is responsible for the synthesis of a number of clinically important bacterial products such as diphtheria toxin, (C. diphtheriae), fibrinolysin (S. aureus), erythrogenic toxin (S. pyogenes), tetanus toxin (C. tetani), botulinum toxin (C. botulinum), and for the serological specificity of the somatic antigens (endotoxins) of Salmonella species and enteropathogenic E. coli. In each case, the bacteriophage genome encodes the genetic information for the synthesis of the specific protein product.

Phages are capable of transduction (phage-mediated gene transfer) and this is probably true for all temperate phages as well as some virulent phages. Transducing phages can pick up DNA from prophages and/or plasmids in donor strains as well as chromosomal DNA and introduce it into appropriate recipient strains. Transduction has been demonstrated to occur in mice by using lysogenic donor and non-lysogenic recipient strains for both S. aureus and E. coli. Transducing phages or their DNA are also taken up by mammalian cells in culture where they persist and/or replicate and in at least one instance express functional gene products.

In closing, it should be noted that there has been increasing evidence over the years to suggest specific relationships between temperate phages and plasmids. Mutant derivatives of λ have been found that fail to integrate into the chromosome but replicate and persist in bacterial cells as extrachromosomal DNA or plasmids. The generalized transducing phage pf16 of Pseudomonas putida, in picking up the genes for degradation of mandelate, was found to acquire the ability to act as a conjugative plasmid and to promote transfer of both

chromosomal genes and genes for mandelate degradation to recipient strains.
The discovery that inheritance of donor genetic markers in intergeneric matings
between E. coli donors and S. typhi recipients and between Klebsiella pneumoniae
donors and E. coli recipients often results in the formation of new plasmids,
raises the question as to the origin of the genes to permit autonomous replica-
tion of these elements. The ubiquity of both defective and non-defective pro-
phages in lysogenic bacteria that should contain such information leads us to
believe that such defective and/or non-defective integrated prophages might
contribute the necessary information for the formation and replication of donor
DNA fragments as autonomously replicating circular plasmid molecules in reci-
pient strains as a consequence of intergeneric matings.

ILLUSTRATIVE EXAMPLES OF EXPERIMENTS IN EACH CLASS

The examples given below are mainly for illustrative purposes. Some of the experiments might not be possible, and there is little or no justification for the performance of certain others.

A. Examples of Class I Experiment:

1. Transductional gene transfer to Escherichia coli using phages P1 or λ from E. coli K-12.
2. Transformation of E. coli K-12 with E. coli K-12 chromosomal, F plasmid or φ80 DNA.
3. Transformation, transduction, or transfection of Bacillus subtilis 168 with B. subtilis 168 chromosomal DNA or PBS1 phage.
4. Transformation of a well-established laboratory strain of Neisseria catarrhalis by DNA derived from the same strain.

B. Examples of Class II Experiment:

1. Conjugal gene transfer between Hfr and F⁻ strains of Salmonella typhimurium LT2.
2. Conjugal gene transfer between Hfr and F⁻ enteropathogenic E. coli strains.
3. Formation of a recombinant plasmid between the pSC101 (tetracycline resistance) and RSF1010 (streptomycin and sulfonamode resistance) plasmids when introduced into E. coli strain K-12.

4. Formation of a recombinant replicon between phage λ and the ColE1 plasmid when introduced into E. coli K-12.
5. Integration of the plasmid R64 into the chromosome of S. typhimurium LT2, and its excision to isolate an R' plasmid.
6. A survey of the host range of R plasmids found in S. typhi strains isolated from nature when introduced into E. coli K-12, S. typhimurium LT2 and Shigella dysenteriae SH.
7. Construction of a recombinant between phage P1 and an ampicillin resistance (Ap) plasmid, and the introduction of the recombinant P1-Ap molecule into E. coli K-12.
8. Construction of a recombinant between bacteriophage Mu and the R plasmid R1drd19 and its introduction into E. coli K-12.
9. Construction of recombinant molecules between phage φ80 and the Col trp (Fredericq) plasmid when introduced into E. coli. (It should be noted that a colicin V gene identical or similar to that on the Fredericq plasmid has been identified in a high proportion of bacterial strains involved in extra-intestinal infection.)
10. Construction of a recombinant DNA molecule involving the plasmid of B. pumilus (carrying genetic information for the inhibition of sporulation) and a temperate phage from B. subtilis when introduced into B. subtilis.
11. Intrageneric transformation of chromosomal DNA in avirulent strains of Streptococci.
12. Intrageneric transformation of chromosomal DNA in Bacillus species except B. anthracis.

13. The introduction of bacteriophage λ into S. typhimurium.

14. Intrageneric chromosome transfer between E. coli K-12 donor and either S. typhimurium, Proteus mirabilis, of Klebsiella aerogenes recipients.

15. Introduction of the genes for nitrogen fixation of the Nif plasmid of K. pneumoneae into recipient strains of E. coli K-12.

C. Examples of Class III Experiment:

1. Construction of a recombinant DNA molecule between the cryptic plasmid from S. typhimurium LT2 and the Staphylococcus aureus plasmid pI258 and its introduction into S. aureus.

2. The introduction of a phage from S. aureus that leads to production of fibrinolysin into a S. albus strain.

3. Construction of recombinant DNA molecules between sea urchin histone genes and a plasmid or bacteriophage replicon from E. coli, and their introduction into E. coli.

4. Construction of recombinant DNA molecules between the Cm plasmid (specifies chloramphenicol resistance) from S. pneumoniae and ColE1, and their introduction into E. coli.

5. Construction of a recombinant DNA molecule between λ or pSC101 and a plasmid derived from Streptomyces coelicolor and its introduction into E. coli.

6. Construction of recombinant DNA molecules between E. coli genes involved in histidine biosynthesis and a B. pumilus plasmid, and their introduction into B. subtilis.

7. Construction of a recombinant plasmid or phage that includes fibroin genes from Bombyx mori, when introduced into E. coli.

8. Construction of a recombinant DNA molecule between the chicken ovalbumin gene and ColE1 and its introduction into E. coli.

9. Construction of a recombinant molecule between the OCT plasmid of Pseudomonas putida and either phage λ or the RSF1010 plasmid, and its introduction into E. coli.

10. Construction of a DNA chimera between mouse mitochondrial DNA and phage λ or the pSC101 plasmid when introduced into E. coli K-12.

D. Examples of Class IV Experiment:

1. Construction of recombinant DNA molecules containing DNA from a phage of S. aureus that codes for the production of fibrinolysin and either E. coli plasmid or phage DNA, and their introduction into E. coli.

2. Construction of recombinant molecules between genes for photosynthesis, derived from any prokaryotic or eukaryotic organism, and E. coli phage or plasmid DNA and their introduction into E. coli.

3. Construction of a recombinant DNA molecule between plasmid DNA (specifying the synthesis of kanamycin) from Streptomyces kanamyceticus and E. coli plasmid or bacteriophage DNA, and its introduction into E. coli.

4. Construction of a recombinant between an S. mutans cariogenic plasmid and an E. coli plasmid and its introduction into E. coli.

5. Construction of a chimeric DNA molecule containing a single purified DNA fragment derived from cucumber mosaic virus and ColE1 and its introduction into E. coli.

6. Construction of a recombinant between the gene coding for the synthesis of human growth hormone and the pSC101 plasmid, and its introduction into E. coli.

E.   Examples of Class V Experiment:

1.   Construction of a recombinant between the S. aureus plasmid that specifies exfoliative toxin production and an E. coli phage or plasmid, and its introduction into E. coli.

2.   Construction of recombinant DNA molecules between cryptic plasmid DNA from microorganisms such as Yersinia pestis, B. anthracis, or Brucella abortus and any other carrier molecule and their introduction into E. coli.

3.   Construction of a chimeric DNA molecule which includes the DNA of 'Dane' particles of the hepatitis B virus and bacteriophage λ or plasmid DNA, and its introduction into E. coli.

F.   Examples of Class VI Experiment:

1.   Construction of a recombinant between the β phage of Corynebacterium diphtheriae that specifies toxin production and a phage or plasmid from E. coli and its introduction into E. coli.

2.   Construction of a recombinant containing genetic information for toxin production from strains of Clostridium botulinum or C. tetani and E. coli phage or plasmid DNA and its introduction into E. coli.

APPENDIX C

GUIDELINES FOR MINIMIZING BIOHAZARDS

A. Introduction

Investigators wishing to construct genetically altered microorganisms should select both the DNA cloning vehicle and the recipient strain with the intent of achieving the greatest possible reduction of known and potential biohazards consistent with the aims of the particular experiment. Whenever possible, the investigators should utilize a recipient-chimera system designed to (1) minimize possible pathogenicity of the genetically altered microorganism; and (2) reduce the likelihood of its dissemination.

These goals may be accomplished by selection of appropriate naturally occurring cloning vehicles and recipient hosts, and by specific genetic manipulation of these vehicles and hosts. The following suggestions may assist in design of experiments, and may permit assignment of a particular experiment to a classification having less stringent levels of containment than might otherwise be possible. We stress that these ideas are offered as guidelines, and not as requirements, since the dictates of any given experiment will determine to a large extent which, if any, of these procedures can be utilized.

B. General Guidance Principles Regarding the Choice of Vehicles for DNA Cloning Experiments

1. By selecting and/or genetically manipulating vehicles used in cloning foreign DNA, investigators may minimize the possible biohazards involved in the construction of genetically altered microorganisms without sacrificing the objectives of the experiment. In general, non-conjugative plasmids are preferable to conjugative plasmids as cloning vehicles.

2. Cloning vehicles which do not offer any biological advantage to recipient bacteria are preferable to vehicles which may offer such an advantage.

3. Cloning vehicles which ordinarily have an intracellular existence are preferable to those existing as encapsulated extracellular particles.

4. Cloning vehicles that express genotypic or phenotypic properties that are already common in the recipient bacterial species are preferable to those expressing less common properties.

5. A vehicle which has not been subjected to experimental procedures, such as mutagenesis, which may alter its biological host range, is preferable to a vehicle which has been subjected to such procedures.

6. Cloning vehicles carrying genetic defects which may restrict their propagation are preferable to wild-type cloning vehicles.

7. Cloning vehicles that have been well characterized with regard to their genetic and molecular properties are preferable to those which have not been as well studied.

C.    General Principles for Use of Antibiotic Resistance Plasmids as Cloning Vehicles

1.  The cloning vehicle selected must not result in introduction of an antibiotic resistance phenotype to a medically important bacterial species in which the resistance phenotype is not found, especially
tic is a drug of choice for the clinical control of the species (e.g., introduction of penicillin resistance into Streptococcus pyogenes or Streptococcus pneumoneae.)

2.  The use of plasmids which carry antibiotic resistance genes that are normally rare in extrachromosomal gene pools (e.g. resistance to trimethoprim and fusidic acid) should be avoided.

3.  Certain antibiotic resistance genes are preferable to others for use as selective agents in DNA cloning experiments; hence, tetracycline, sulfonamide, and streptomycin resistance are preferable for use because they occur naturally at high frequency among microorganisms present in both human and domestic animal populations.

D.  Guidelines for Selection of Bacteria as DNA Donors and Recipients

1.  Hosts that possess conjugative plasmids or prophages, which may facilitate dissemination of genetic material to other hosts, should be avoided if consistent with the objectives of the experiment.

2.  When little is known about the genetic, metabolic, and/or ecological properties of a donor or recipient strain, such strains should be avoided for construction of genetically altered microorganisms.

3.  Spore-forming microorganisms should not be used as donors or recipients of chimeric DNA molecules; mutant derivatives unable to form spores should be employed; restoration of sporogeny should not be a possible outcome of the experiment.

E.  Suggestions for Possible Genetic Modification of Recipient Strains

Genetic modification of the recipient strains prior to introduction of recombinant DNA molecules may contribute further to reducing or eliminating possible biohazards.  The use of recipient strains that possess mutations that reduce pathogenicity, ability to survive and/or establish in a diversity of ecological habitats and/or transmit genetic information is therefore desirable. Examples of genetic modifications that can be introduced into E. coli strains to accomplish the above objectives are provided below:

1.  Use of a pur⁻ mutant since purine-deficient mutants of many pathogenic microorganisms are avirulent.
2.  Use of a dap⁻ mutant since the amino acid diaminopimelic acid is not very prevalent in natural environments and its absence will result in inability to synthesize the cell wall and thus lead to cell lysis.
3.  Use of a temperature-sensitive mutant that cannot grow at 37°C.  This would minimize the ability of the genetically altered microorganism to colonize animal hosts.
4.  Use of a cold-sensitive mutant that cannot grow at temperatures below 32°C.  This would minimize the ability of the genetically altered microorganism to survive in soil, water and other natural environments.

5.  Use of a strain that would be unable to ferment or utilize a diversity of carbohydrates - e.g. a pts mutant, phosphotransferase system defective.  This would contribute to the inability of the genetically altered microorganism to grow in a diversity of ecological habitats.

6.  Use of a mutant with mutations such as uvr, polA, etc. that would confer increased sensitivity to ultraviolet light, since this would contribute to inability of the genetically altered microorganism to survive in natural environments.

7.  Use of a rec⁻ mutant since this might reduce the exchange of genetic information by the recipient strain.

8.  Use of a bacterial mutant that is deficient as a recipient of genetic information by conjugation.  This would reduce the likelihood of introduction of conjugative plasmids from other bacteria in the natural environments and thus reduce the likelihood of mobilization and transmission of the information on the recombinant DNA molecule by conjugation.  Some mutations that inhibit conjugation by bacteria may also confer increased resistance to a diversity of bacteriophages, and thus might reduce the likelihood of transmission of genetic information by transduction.

9.  Use of a mutant that is resistant to a multitude of potential transducing phages since this would minimize the likelihood of dissemination of genetic information from the genetically altered microorganism.

APPENDIX D

GUIDELINES FOR MONITORING AND REASSESSMENT OF BIOHAZARDS ASSOCIATED WITH
RECOMBINANT DNA MOLECULES INTRODUCED INTO MICROORGANISMS

A. Introduction

After construction of a recombinant DNA molecule and its introduction into
a microbial host, it will be important for the investigator to assess the real
biohazards associated with the formation of this genetically altered microorganism.
In many instances the information obtained from these studies will require reclas-
sification of the experiment into a new class category. Reclassification might
result in the experiment being designated in a class requiring less contain-
ment, although in certain circumstances the determined biohazards may be more
severe than originally expected which would require the reclassification of the
experiment into a class requiring a more stringent level of containment.

Certain principles should be followed in obtaining information that might
be useful in assessing the real biohazards associated with any given experi-
ment. One should initially conduct specific experiments to determine whether
there are any alterations in the pathogenicity of the genetically altered micro-
organism and any changes in its ecological potentials. If the altered micro-
organism contains DNA specifying unknown gene products it will be difficult,
if not impossible, to assess the biohazards associated with the distribution of
this genetic information among microorganisms occupying the same ecological
niches as the recipient strain. In these instances it will not be possible to
reclassify the experiment to employ less stringent degrees of containment. In
these evaluation experiments, the cells containing recombinant DNA should be
grown under the same conditions of containment as were used in the experiments
that produced them. If cell products are to be analyzed, the cells should be
lysed or extracted under these same conditions and these extracts tested for
sterility prior to taking the material into a general research laboratory
where less containment is necessary. If the product is potentially toxic, then
appropriate precautions need to be taken to protect the investigator from
exposure, and special facilities should be utilized to house any animals and/or
plants used for testing the product. When the genetically altered microorganisms
are being evaluated for pathogenicity in animal or plant hosts, these animals
or plants should be under containment facilities similar to those used for
the construction of the genetically altered microorganism. Such animal or
plant hosts must be disposed of in a way that will not permit dissemination
of the organism being tested. Tests requiring large numbers of altered micro-
organisms should be avoided if possible until there has been some assessment of
the biohazard. If this is not possible, then such experiments should be conduc-
ted under conditions of more stringent containment.

B. Information That Will Be Helpful in Evaluating Pathogenicity

The following tests should not be considered to be all-inclusive since the
particular tests to be performed will be dictated by the nature of the geneti-
cally altered microorganism, with respect to both the origin of the genetic
information on the recombinant DNA molecule and the particular attributes of the
recipient host species. The design and conduct of specific experiments to
evaluate the real biohazards will therefore require careful evaluation by the

investigator. Some of the relevant types of experiments that can be conducted on the genetically altered microorganism include determination of its properties in the following tests:

1. Infectivity in appropriate animals or plants.
2. Colonization in the gut, oral cavity, on the skin, etc. of model animal hosts or on the roots, leaves, etc. of appropriate plants.
3. Production of keratoconjunctivitis in guinea pigs (the Sereny test) which would be an indication of the capacity of the altered microorganism to penetrate the intestinal mucosa.
4. Invasion and proliferation in macrophages and/or fibroblasts.
5. Production of such cell products as bacteriocins, hemolysins, fibrinolysins, collagenases, pectinases, etc. that might contribute to colonizing ability and/or invasiveness and toxins of various sorts and to test the potency of such toxins by using appropriate cell cultures of eukaryotic organisms, ligated intestinal loops of appropriate animal hosts or appropriate plant or animal species.
6. Production of hypersensitivity and/or necrosis by cells or extracts when injected intradermally into the skin of appropriate animal hosts.
7. Determination of the minimal inhibitory concentrations of various antimicrobial agents useful in killing and/or inhibiting growth of the altered microorganism.
8. Determination of whether or not the gene products specified by the recombinant DNA appear extracellularly, intracellularly or in the periplasmic space.

## C.  Information That Will Be Helpful in Evaluating Ecological Potential

The individual experiments needed to assess ecological potential of the altered microorganism will of necessity be dictated by the properties of the strains used to construct it. The following types of experiments should therefore only serve to illustrate the range of tests to determine the properties of the genetically altered microorganism:

1. Expression of the genetic traits that are specified by the recombinant DNA molecule.
2. Resistance to UV, disinfectants, etc.
3. Survival in soil, water and the dry state or in any ecological habitat likely to be occupied.
4. Ability to form spores.
5. General metabolic activities and attributes including changes in growth rate, utilizable and preferred substrates, temperature and pH optima for growth, aerobic vs. anaerobic growth, photosynthetic and $N_2$ fixing ability, etc.
6. Production of substances that displace or inhibit other microorganisms that normally occupy the same ecological habitats.

## D.  Other Information Needed to Evaluate the Severity of Biohazards

It will be extremely important to test the ability of the recombinant DNA contained in the altered microorganism to be transmitted by phage and/or

conjugative plasmids to other strains of the same species as the recipient as well as to other species of bacteria known to exchange genetic information with the recipient host species. Such tests should also be performed with other strains of the bacterial species from which DNA was obtained to construct the recombinant DNA, even when these species are not known to exchange genetic information with each other. Since some microbial species are known to excrete DNA into the medium which is sometimes biologically active, tests should also be done to determine whether the recombinant DNA is capable of being taken up and expressed in other microorganisms by transformation. Such tests for examining transmission of the recombinant DNA by transduction, transfection, conjugation, transformation and/or by encapsulation of the recombinant DNA in phage virions should be tested _in vitro_ experiments and in some instances under _in vivo_ conditions with appropriate animal and/or plant hosts.

E. Summary

If one performs any or all of the above experimental tests to evaluate potential biohazards of genetically altered microorganisms, it will be necessary to include as controls the organisms used as donors of the genetic information to form the recombinant DNA molecule as well as the recipient host strain.