Molecular Breeding and Genetics of Applied Microorganisms

Edited by
Kenji Sakaguchi
Masanori Okanishi

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Edited by

Kenji SAKAGUCHI

Mitsubishi-Kasei Institute of Life Science, Tokyo

and

Masanori OKANISHI

National Institution of Health, Tokyo

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Contributors

Numbers in parentheses indicate the chapters (or sections) to which the authors' contributions submit.

Teruhiko BEPPU (5.1, 8.4), Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, *Bunkyo-ku, Tokyo 113, Japan*

Akira FURUYA (9.2), Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Ltd., *Machida-shi, Tokyo 194, Japan*

Norio GUNGE (7.3), Laboratory of Microbiological Chemistry, Mitsubishi-Kasei Institute of Life Science, *Machida-shi, Tokyo 194, Japan*

Sueharu HORINOUCHI (8.4), Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, *Bunkyo-ku, Tokyo 113, Japan*

Kunimoto HOTTA (2), Institute of Microbial Chemistry, *Shinagawa-ku, Tokyo 141, Japan*

Yo KIKUCHI (6), Laboratory of Microbiological Chemistry, Mitsubishi-Kasei Institute of Life Science, *Machida-shi, Tokyo 194, Japan*

Bunji MARUO (8.1.3), Department of Agricultural Chemistry, Nihon University, *Setagaya-ku, Tokyo 154, Japan*

Haruo MOMOSE (9.1), Tokyo Research Laboratory, Ajinomoto Co. Inc., *Kawasaki-ku, Kawasaki-shi 194, Japan*

Kenji NAGAHARI (8.2), Laboratory of Microbiological Chemistry, Mitsubishi-Kasei Institute of Life Science, *Machida-shi, Tokyo 194, Japan*

Shigetada NAKANISHI (5.2), Department of Medical Chemistry, Faculty of Medicine, Kyoto University, *Sakyo-ku, Kyoto 606, Japan*

Shosaku NUMA (5.2), Department of Medical Chemistry, Faculty of Medicine, Kyoto University, *Sakyo-ku, Kyoto 606, Japan*

Kozo OCHI (7.2), Laboratory of Molecular Biology, National Institute of Neurological Diseases and Stroke, N.I.H., *Bethesda, Md. 20014, U.S.A.*

Masanori OKANISHI (2,3), National Institute of Health, *Shinagawa-ku, Tokyo 141, Japan*

Hiuga SAITO (8.1.2), Institute of Applied Microbiology, University of Tokyo, *Bunkyo-ku, Tokyo 113, Japan*

Kenji SAKAGUCHI (Introduction, 1, 7.1, 8.1.1, 8.3), Laboratory of Microbiological Chemistry, Mitsubishi-Kasei Institute of Life Science, *Machida-shi, Tokyo 194, Japan*

Kazuo UCHIDA (7.4), Research Laboratory, Yamasa Shoyu Co. Ltd., *Choshi-shi 288, Japan*

Hiroo UCHIYAMA (5.1), Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, *Bunkyo-ku, Tokyo 113, Japan*

Takeshi UOZUMI (5.1, 8.4), Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, *Bunkyo-ku, Tokyo 113, Japan*

Kunio YAMANE (8.1.3), Institute of Applied Microbiology, University of Tokyo, *Bunkyo-ku, Tokyo 113, Japan*

Keiji YANO (4), Department of Radiation Microbiology, Faculty of Agriculture, University of Tokyo, *Bunkyo-ku, Tokyo 113, Japan*

Preface

Applications of microbiology have long history in Japanese industry, beginning in the traditional brewing industries which use various unique microorganisms. By the late 1950's, the industrial production of antibiotics and amino acids by fermentative processes was beginning in Japan, and this was rapidly followed by the microbial production of nucleic acid-related compounds, physiologically active substances and many other materials with each different organisms. However, the revolutionary developments in molecular biology have raised many new possibilities, and the new genetic techniques to recombine genetic materials of far different organisms are expected to promote the progress of applied microbiology extensively. In this publication, recent achievements in this field in Japan are reviewed by many contributors of each specialities.

The editors extend grateful thanks to the scientists who have contributed to and made possible the publication of this volume.

Kenji SAKAGUCHI
Masanori OKANISHI

Introduction*

In this newly developed field, some of the specific areas where the editors feel that further progress is possible especially in applied microbiology are outlined below.

1. In basic research. It is possible to accumulate a specific gene for studies of its structure, of the protein translated from it and of its regulatory mechanism. The use of recombinant DNA techniques makes it possible to link fragments of DNA from unrelated organisms for study. These techniques have been applied to study cancer, immune reactions, the replication site of DNA, etc., and form essential tools for modern biological studies.

2. In pollution control. Bacteria belonging to *Pseudomonas* have been created which scavenge petroleum wastes effectively. It should be possible to create microbes that can metabolize sewage, phenol, cyanide, organic acids and other wastes from cities and factories. In particular, it may be possible to create a microbe able to metabolize organic mercury compounds.

3. This technology is expected to improve fermentative production processes in the antibiotics industry, as well as other fermentation industries, such as the production of amino acids, inosinic acid, enzymes, etc.

4. Studies to produce hydrogen gas from water or to construct a "biological battery" using a photosynthetic system are in progress; such devices would be pollution-free. The construction of improved methane bacteria would also be useful.

5. Increased efficiency of agricultural production may be possible by breeding plants with blocked photorespiratory pathways, and having higher carbon dioxide-assimilating ability.

* Kenji SAKAGUCHI, Mitsubishi-Kasei Institute of Life Science.

6. Studies to introduce nitrogen-fixing genes into either bacteria living around the roots of plants or into plants themselves are important. There are many difficulties, but the discovery of potent organisms fixing nitrogen and improved techniques of protoplast fusion are expected to lead to success. Breeding bacteria for the rapid formation of "natural fertilizer" may be another possibility.

7. The breeding of plants by combinations of protoplast fusion and DNA transformation should be more effective than traditional methods, because the former technique is not seasonal and can work between very different species. It should be possible to breed species tolerant to various plant diseases.

8. The production of physiologically active peptides such as insulin or somatostatin has already been achieved. The production of antibodies, interferon and others should also be possible.

9. It may be possible to produce protein of good quality for use as food by transferring genes from plants or animals into yeasts or other microbes.

10. The medical applications of these techniques will be important in the longer term.

However, the dangers of these techniques must not be overlooked especially on pathogenic bacteria or viruses. Many countries already have regulations governing the handling of potentially pathogenic or biohazardous organisms. It is disirable to investigate the possible pathogens in proper physical or biological containments. In addition, proper facilities or institutions for risk assessment, national or international, are required to promote both academic and practical studies in this field. On the other hand, almost all hybridized microorganisms produced from non-pathogens are likely to be safe, and the development of self-cloning systems and recombinants among non-pathogenic microorganisms should make practical large-scale cultivation for many purposes both safe and feasible.

Contents

CHAPTER 1

Species Barriers to the Maintenance and Expression of Foreign DNA*

This chapter reviews the species barriers against the proliferation and expression of genetic material originating from foreign organisms. These barriers can be classified into six categories, the nuclease barrier, the replication unit barrier, the transcription barrier, the translation barrier, the proteinase barrier and the intervening gene barrier.

Many attempts to introduce DNA of taxonomically distant bacteria,[1-5] yeasts[6-8] and higher organisms[9-14] into *E. coli* cells by forming hybrid DNA molecules with *E. coli* plasmids have been made. The *Bacillus subtilis leu* gene was successfully introduced into *E. coli leu*$^-$ cells, giving rise to *leu*$^+$ transformants.[4] However, attempts to introduce the *E. coli leu, trp* or ampicillin resistance gene into *B. subtilis* have met with consistent failure (ref. 4, and unpublished results). The only eukaryotic genes which have so far been reported to be expressed in *E. coli* cells, not using the complementary DNA technique, are the *Saccharomyces cerevisiae his*,[6] *leu, trp*[7] and mitochondrial[8] genes. Other eukaryotic genes introduced into *E. coli* from *Drosophila* chromosomes,[10,12] sea urchin histone genes,[11] mouse mitochondria,[13] DNA copies of rabbit globin mRNA,[14] or *Xenopus laevis* DNA[9] were sometimes reported to produce RNA, but neither enzymes

* Kenji SAKAGUCHI, Mitsubishi-Kasei Institute of Life Science.

nor proteins immunologically cross-reacting with the original gene products were observed.

1.1 Barriers to the Genetic Establishment and Expression of Foreign DNA

1.1.1 The Nuclease Barrier

Table 1.1 summarizes the postulated barriers, with examples. The nuclease barrier involves the degradation of the genetic principles introduced in the growth medium, on the cell surface, and in the cytoplasm. Of course, microorganisms which produce nucleases in the culture medium, for instance, some strains of the *B. subtilis* group, many strains belonging to *Actinomycetales* and *Clostridium*, and many fungi, are not expected to be transformable without further treatments. Even washed cells have various nucleolytic enzymes in the membrane and in the peripheral area of double-layered membranes, including DNase I in *E. coli*.[15] They are presumed to provide protection against invading foreign genetic materials. Seto *et al.*[16] reported membrane-bound nucleolytic activity in *Diplococcus pneumoniae* cells and the degradation and rejection of incoming exogenous DNA.

Genetic materials introduced into a cytoplasmic environment suffer further degradation by the cytoplasmic nucleases. Three different nuclease systems acting against introduced DNA are already known. The intracellular inactivation reported in the case of transfecting bacteriophage ϕ82G DNA into *B. subtilis* cells was caused by nucleases different from restriction enzymes within the cell, because the phage was developed in the same strain.[17]

The restriction endonucleases act to prevent the invasion of DNA from foreign organisms, allowing only the propagation of native DNA (Table 1.1). In order to obtain the same number of transformants, over

TABLE 1.1 The Nuclease Barrier

	Restriction endonuclease	
	DNA required (μg per transformant)	
	E. coli C600 $r_k^- m_k^-$ *leu*$^-$	*E. coli* C600 *leu*$^-$
RSF2124-B-Leu†	9.0×10^{-6} μg	> 1 μg
RSF1010-B-Leu†	3.5×10^{-6} μg	> 1 μg

† These hybrid plasmids contain *leu* gene obtained from *Bacillus subtilis* chromosome.[4]

10^5 times more transforming DNA is necessary in *E. coli* wild strain than in an *Eco* K restriction enzyme-deficient mutant.

The *E. coli recB* and *C* mutants were reported to be transformed by linear DNA but wild cells were transformed only by covalently closed circular DNA since the *recB* and *C* genes were essential for the production of exonuclease in *E. coli* cells.[18]

1.1.2 The Replication Unit Barrier

The transformation of *E. coli* plasmids, pSC101 and RSF1010 plasmids, into a *B. subtilis* restriction—deficient mutant was attempted but failed consistently[19] (unpublished results). Leavitt *et al.*[20] reported that the labelled bacteriophage T4 DNA could penetrate and remain for several generations in Syrian hamster embryo cells, but then disappeared within several generations. The replication machinery is complex, and it is now evident that at least several enzymes participate even in the simple Col El DNA replication system, which utilizes only DNA polymerase I in the polymerization reaction. This system includes DNA sequence specificity at the origin and at other possible specific sequences which may interact with RNA polymerase,[21] gyrase,[22] etc. It forms a strong barrier to the proliferation of foreign DNA in the cell.

1.1.3 The Transcription Barrier

Several promoter sequences in *E. coli* and its phage DNA have been determined, including tyrosine tRNA,[23] *lac* promoter and operator region,[24] phage λ gene *N* promoter[25] and phage fd DNA.[26] The sequences were all different, probably reflecting the strength of interactions with RNA polymerases and with other regulatory proteins and their effectors. Within phage λ DNA, a strong promoter site exists on top of the S gene which acts only on the RNA polymerase modified by Q gene product and initiates vigorous synthesis of coat and tail protein mRNA.[27] Phage T7 DNA has a gene for its own RNA polymerase of molecular weight ca. 100,000 and transcribes the major part of its DNA.[28] *Achlya* RNA polymerases were divided into six different forms and it was shown that their actions on the signalling compounds HS 1, 2, 3 are different; inactivating or derepressing. The polymerases transcribe *Achlya* DNA much efficiently than calf thymus DNA, and no transcription occurred on poly (dAT).[29] Animal and plant cells have their own complicated RNA polymerase systems, which are different from each other.[30] These observations indicate that the specificity of attachment at the promoter site can vary with every combination of RNA polymerase and DNA sequence. Many illegitimate enzymes

can bind only weakly or not at all to promoter sites of different DNAs. Knowledges are accumulating about the terminating sequence of transcription. The attenuator site on the leader region of the tryptophan operon in *E. coli* has been sequenced.[31] it is known that RNA polymerase has a specific termination site, and failure to recognize a specific termination site may result in the production of an inactive protein.

1.1.4 The Translation Barrier

This barrier is probably one of the strongest. In introducing animal or plant DNA into *E. coli* by plasmid techniques, RNAs were produced from sea urchin histone DNA,[11] *Xenopus laevis* DNA[9] and *Drosophila melanogaster* DNA,[10,12] but no proteins could be detected by immunological inspection or by enzyme assay. Leffler and Szer[32] introduced *E. coli* MS2 RNA phage RNA into an *in vitro* translation system consisting of *Caulobacter crescentus* and *Bacillus brevis* ribosomal and other factors, and no incorporation of [^{14}C]lysine into acid-precipitable polymers was observed. They found that the 30S ribosomal particle was responsible for this specificity.

These phenomena are comprehensible in the light of the fact that 16S ribosomal RNA should have a sequence complementary to that of its proper mRNA.[33,34] The initiation factors have species differences[35,36] and their binding specificity for ribosomes or RNAs are probably different; thus there may be strict specificity of mRNA secondary structure for binding to the ribosome to initiate peptide synthesis.[37]

1.1.5 The Proteinase Barrier

There are many kinds of intracellular proteinases which are usually inert towards well-conformed cell proteins. When an unusual protein produced from foreign DNA appears in the cell, the proteinase may attack the unfamiliar conformations.

1.1.6 The Intervening Gene Barrier

The existence of intervening genes or introns in the eukaryotic gene construction has been reported in the genes of mouse immunoglobulin,[38] ovomucoid,[39] SV40 virus[40] and others. Splicing enzymes are not native to prokaryotic cells. It is known that the direct introduction of eukaryotic genes into prokaryotes gives non-functional protein products.

The existence of these species barriers suggests that recombinant DNA may represent a lower potential hazard than natural pathogenic or-

TABLE 1.2 Species Barriers

1. NUCLEASE BARRIER
   SURFACE DEGRADATION AND RELEASE. Pneumococcal transformation (H. Seto *et al.*, 1975)
   INTRACELLULAR INACTIVATION. SP82 G DNA suffers about 40 lesions when transfected into *Bacillus subtilis* cell (D. M. Green *et al.*, 1968).
   RESTRICTION ENDONUCLEASES. Over 10^5 times more DNA is necessary in transforming *E. coli* $r_k^+m_k^+$ cells comparing to the restriction-deficient cells (K. Nagahari & K. Sakaguchi).
2. REPLICATION UNIT BARRIER
   Inability to introduce *E. coli* plasmids PSC101, RSF1010 into *B. subtilis* r^-m^- cells (T. Tanaka & K. Sakaguchi). Degradation of phage T7 DNA after penetration into Syrian hamster embryonic cells (J. C. Leavitt *et al.*, 1974).
3. TRANSCRIPTION BARRIER
   PROMOTER SPECIFICITY. Molecular differences of RNA polymerase in bacteria, phages, eukaryotic cells. Exchange of σ factor with σ^λ in phage λ-infected *E. coli*. Difference of DNA sequence on each promoter.
   TERMINATION POINT SPECIFICITY.
4. TRANSLATION BARRIER
   BINDING SPECIFICITY OF RIBOSOMES. *Bacillus brevis* or *Caulobacter crescentus* 30S subunit does not bind to MS2 RNA (S. Leffler and W. Szer, 1974). Inability to form histone protein from sea urchin histone genes in *E. coli* minicells (L. H. Kedes *et al.*, 1975).
   SIMILAR OBSERVATIONS: *Xenopus laevis*, *Drospophila melanogaster*, mouse mitochondrial DNA.
5. PROTEINASE BARRIER
   Intracellular proteinase may destroy unfamiliar or poorly conformed proteins produced in the cell. For instance, the peptides produced from synthesized DNA, eukaryotic protein in bacteria, etc.
6. INTERVENING GENE BARRIER
   Many eukaryotic organisms have a gene construction with intervening genes. Such genes do not form normal protein when they are introduced into prokaryotes.

ganisms or even virus particles, especially in cases of hybridization with distantly related organisms, such as hybridization of animal DNA with that of plant or microorganisms or *vice versa*. The matching of replicational, transcriptional and translational machinery between taxonomically different organisms is now becoming to be possible,[41,42] but the "patching" of host initiation or termination sites and others necessary.

1.2 Selective Disadvantage of Hosts Harboring Recombinant Molecules

Nagahari found that RSF1010 trp hybrid plasmid carrying *E. coli trp* operon produced over two hundred times more *E. coli*-type tryptophan synthetase in *Pseudomonas* cells. However, after overnight culture, 90% of the surviving cells had lost the plasmid.[43]

A point to be stressed is that host microorganisms which carry a composite plasmid constructed *in vitro* should not be able to become predominant in a natural environment because they are forced to produce unnecessary enzymes (in this case, antibotic-inactivating enzymes and tryptophan—synthesizing enzymes), suppressing the production of normal enzymes necessary for rapid growth under natural circumstances. They thus have a selective disadvantage. Microorganisms that have acquired composite plasmids by conjugation also have the same disadvantages under natural conditions.

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