Industrial Aspects of Biochemistry and Genetics

Edited by
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NATO ASI Series

Series A: Life Sciences Vol. 87

Industrial Aspects of Biochemistry and Genetics

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Plenum Press
New York and London
Published in cooperation with NATO Scientific Affairs Division

Proceedings of a NATO Advanced Study Institute on
Industrial Aspects of Biochemistry and Genetics,
held September 4–14, 1983,
in Cesme, Turkey

Library of Congress Cataloging in Publication Data

NATO Advanced Study Institute on Industrial Aspects of Biochemistry and Genetics (1983: Çesme, Turkey)
Industrial aspects of biochemistry and genetics.

(NATO ASI series. Series A, Life sciences; vol. 87)
"Proceedings of a NATO Advanced Study Institute on Industrial Aspects of Biochemistry and Genetics, held September 4–14, 1983, in Cesme, Turkey"—T.p. verso.
"Published in cooperation with NATO Scientific Affairs Division."
Bibliography: p.
Includes index.
1. Industrial microbiology—Congresses. 2. Biochemical engineering—Congresses. 3. Microbial genetics—Congresses. I. Alaeddinoğlu, N. Gürdal. II. North Atlantic Treaty Organization. Scientific Affairs Division. III. Title. IV. Series: NATO ASI series. Series A, Life sciences; v. 87.
QR53.N37 1983 660.6 85-3690
ISBN 0-306-41934-3

A Division of Plenum Publishing Corporation
233 Spring Street, New York, N.Y. 10013

Printed in the United States of America

Industrial Aspects of Biochemistry and Genetics

NATO ASI Series

Advanced Science Institutes Series

A series presenting the results of activities sponsored by the NATO Science Committee, which aims at the dissemination of advanced scientific and technological knowledge, with a view to strengthening links between scientific communities.

The series is published by an international board of publishers in conjunction with the NATO Scientific Affairs Division

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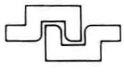

Series A: Life Sciences

PREFACE

This book includes the proceedings of a NATO Advanced Study Institute held at Cesme (Turkey), from September 4 to 14, 1983.

Recent advances in molecular biology have generated a wave of excitement about the prospective application of novel microbiological techniques in a wide range of industrial roles. The new methodology, brought about by recombinant DNA technology, has given the biologist direct access to the genome which in turn, envisages the adaption of certain microorganisms to industrial production, much higher yields of such products as antibiotics and enzymes by biotechnological industry, and the industrial production of substances which occur in nature in very low concentrations and cannot be economically recovered.

In the chapters that follow, the accomplishments and the promise of genetic engineering and industrial microbiology are set forth by its practitioners.

We greatly acknowledge the help of those in preparation of the individual manuscripts and our thanks to all participants of the meeting for their particular efforts.

Nafi G Alaeddinoğlu
Arnold L Demain
Giancarlo C Lancini

CONTENTS

PLASMID INCOMPATIBILITY AND REPLICATION

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Plasmids are covalently closed circular DNA molecules that replicate autonomously in bacteria. Because of their small size and relative simplicity, their replication was studied extensively as a model for the replication of the complex cell chromosome. Recently, plasmids have gained more attention because they are the most commonly used cloning vehicles in genetic engineering experiments. The earliest gene cloning experiments were done solely for scientific interest, but soon the contribution of gene cloning techniques to industry was realized and are now being used extensively in biotechnology.

Some plasmids, namely the R factors, confer antibiotic resistance to the host bacterium. These plasmids are important in the epidemiology of antibiotic resistant pathogens of both man and animals. A special property of some of the R factors is self-transmissibility from the host to another bacterium. This property results in the spread of antibiotic resistance among bacterial pathogens (1).

Studying the relatedness of plasmids in pathogenic bacteria can reveal the epidemiology of drug resistant clinical isolates. Relatedness of plasmids can now be studied by DNA homology, but the faster and simpler traditional method has been by examining incompatibility properties. Two plasmids are said to be incompatible if they cannot be stably inherited in a cell line. If a bacterium is transformed with two such plasmids simultaneously, after some cell divisions, each bacterium in the culture will harbor only one or the other plasmid, but not both. This is the result of competition for similar factors necessary for replication and maintenance. Plasmids can be classified by placing those which are incompatible in the same group. Incompatible plasmids indeed exhibit similar replication properties such as copy number and have DNA sequences in common. However, since a plasmid

may be incompatible with members of more than one group, classification of plasmids by incompatibility properties is an oversimplification. Nevertheless, this classification gives a broad idea about plasmid replication and is important in studies of the spread of clinically important plasmids. Incompatibility properties of mainly the E.coli plasmids have been studied in detail. These plasmids constitute about 25 main incompatibility groups, and members of each group exhibit properties similar with each other, but quite different than those of other groups (1). Studies of the mechanisms of incompatibility have generated much information about plasmid maintenance which has been useful for the construction of cloning vehicles. The general features of maintenance which will be discussed are stable inheritance/partitioning and autonomous replication with a defined copy number.

At least for low copy number plasmids, a partitioning mechanism exists ensuring that each new cell inherits at least one copy of the plasmid. For high copy number plasmids segregation to daughter cells may not need any active partitioning mechanism. Because there are a large number of plasmid copies in a cell, the probability for each daughter cell to inherit at least one copy is high. However, this random segregation model is not feasible for low copy number plasmids. For example F plasmid which exists as 1-2 copies per cell, is very stable in a cell line. That degree of stability would not be expected if segregationn were random. The alternative model to random segregation is regulated segregation, or equipartition which presumes that an active mechanism ensures that each daughter cell inherits at least one copy of the plasmid. Since F plasmid partitioning is so finely controlled, an obvious assumption is that its partitioning is controlled by a mechanism similar to that of the cell chromosome. The present data is not sufficient to clarify this point. The equipartition model presumes the existance of partitioning genes; the incD region of F plasmid may provide this function. A region of DNA is considered to be an incompatibility region (inc) if it prevents the maintenance of the parent plasmid in the same cell when it is joined to an unrelated plasmid. Mini F is a 9 kilobase (kb) EcoRI fragment of the 94.5 kb F plasmid which is capable of autonomous replication; it also exhibits the same replication properties of F plasmid (2,3). This method of dissection of plasmids to smaller autonomously replicating fragments has been very useful for studying DNA replication. IncD region is not essential for plasmid replication, but its deletion results in an unstable plasmid (4). Among the three inc regions of F, incD expresses the weakest incompatibility against F plasmids (5). IncD is in the region common to other plasmids in the FI incompatibility group, as has been shown by DNA heteroduplex mapping using electron microscopy (6). It is also the only inc region that expresses incompatibility against those plasmids (7).

To find out the nature of the partitioning gene, Ogura and Hiraga mapped three separate genes in the incD region (8). SopA (sop:stabi-

lity of plasmid) and sopB act in trans, but sopC functions only in cis, suggesting that it is the specific site necessary for actual partitioning. The data also suggest that the proteins coded by sopA and sopB may act on sopC. Hayakawa and Matsubara have found that the sopB protein binds around sopC; two presumably host-coded proteins bind sopC concomitantly. The sopC gene product in conjunction with these host proteins may therefore bind to the partitioning site on cell membrane. Irrespective of whether the proposed model proves to be correct or not, the present data on incD offers strong evidence that partitioning genes exists, and one way that plasmid incompatibility arises is by competition for stability.

The only other inc regions yet described, incB and incC, play a role in replication and copy number control (9). It contributes greatly to incompatibility by keeping the total number of copies fixed. If in the same cell there are two similar plasmids, A and B, each having a copy number of two, then after plasmid replication and cell division each daughter cell will inherit any two copies. Since it cannot distinguish between the two plasmids, two possibilities with equal probability emerge: the two daughter cells harbor two copies of one plasmid or the two daughter cells harbor one of each plasmid. For the latter cells, there is again only 50% chance that they will give rise to cells with mixed plasmids. Therefore, eventually each cell in the culture will harbor only one or the other plasmid. For higher copy number plasmids the picture is similar, but of course more cell doublings are necessary to reach the pure plasmid state.

Two models have been proposed to explain how the copy number can be kept at a particular level. The positive control model proposed by Jacob et.al. (10) suggests that the number of copies of a plasmid is determined by the limited amount of a factor (or factors) necessary for replication. Alternatively, the negative control model proposed by Pritchard et.al. (13) assumes that there is a diffusable inhibitor of replication that acts in trans and is coded by the plasmid. The concentration of the inhibitor is proportional to the number of plasmids in the cell, and is diluted out as the cell mass increases before cell division. The currently available data suggest that a number of plasmids such as ColE1 employ the latter model. A 555 nucleotide long primer RNA for initiation of ColE1 DNA replication is processed by RNAseH from the primer precursor transcribed from the region to the right of the origin of DNA replication (ori). A 108 nucleotide long transcript synthesized from an overlapping segment of the complementary strand is partially homologous to primer RNA. This RNA I transcript interacts with primer RNA to inhibit the initiation of DNA replication (12). The rop gene located to the left of ori also regulates DNA replication by inhibiting the initiation of transcription of primer RNA (13). This gene codes for a 63 amino acid protein which is not essential for replication. Of the three genes, only RNAI influences incompatibility. Lacetena and Cesareni (14) and Tomizawa and Itoh (15) showed independently that inhibition is due to base

pairing of RNAI with the primer RNA, thus inhibiting base pairing of the primer with the DNA. Mutations which reduce the homology (hybridization) between RNAI and the primer RNA increase the availability of primer RNA for the initiation of DNA replication. This results in a decrease in the expression of incompatibility and an increase in copy number.

It is not known why two negative control systems, RNAI and the rop gene, have evolved in ColE1 plasmid. It is interesting that of these two genes only the RNAI plays a role in the expression of incompatibility. ColE1 replication is nevertheless a simple system that does not even require any proteins coded by the plasmid, and it exhibits probably the simplest incompatibility mechanism.

For larger plasmids, the picture seems much more complicated, and not yet clear. For example, for plasmids RI (a large plasmid with low copy number), two regions (cop) control the copy number but only copA expresses incompatibility. The product of this gene was shown by Stougaard et al (16) to be a 90 nucleotide long unstable RNA, with a half life of less than a few minutes. The nucleotide sequence analysis revealed that this RNA is like RNAI of ColE1 in that it is untranslatable, but has potential for high degree of secondary structure. Light and Molin (17) showed that the target of this RNA lies between the promoter and a gene which is required for replication (repA). Thus copA RNA prevents expression of repA gene, either by interfering with translation or with transcription. The mechanism of action of this RNA is therefore very different than that of ColE1 RNAI, in spite of the similarity of function.

Our present knowledge of F plasmid replication is limited. As previously mentioned, copy number of this plasmid is strictly controlled. Between the regions incB and incC is the gene for the 29K protein essential for replication (18). Kline (16) has shown that disruption of the incC region by transposon insertion results in high copy number. Five 22 base pair (bp) direct repeats are found in the nucleotide sequence of this region (18, 19). The presence of four similar 19-22 bp repeats in incB was revealed by Murotsu et.al. (18). When cloned into another plasmid, a 58 bp fragment of incC containing two of the repeats expresses incompatibility against F plasmid. Two such fragments cloned in tandem express much stronger incompatibility (19). What these repeats do at the molecular level is still not known, but similar repeats are observed in the replication regions of other plasmids as well as bacteriophage lambda (figure 1). These are the repeats in the region essential for replication of pR6K, a 38 kb plasmid that specifies resistance to antibiotics ampicillin and streptomycin. The repeat indicated by asterisk is in the promoter region for the protein that initiates DNA replication in vitro (20). The sequence analysis has revealed the presence of eight similar repeats in pRK2 (21). RK2 is a 56 kb plasmid with copy number of seven and is able to replicate in a variety of hosts. The repeats have a common core

sequence, a hexanucleotide, except in the case of pR6K where G has been replaced by the other purine. The core sequence is also found in the lambda phage replication region (22). It is also found once in the replication region of E.coli chromosome (23,24). Some plasmids such as RI, however, do not contain repeats in replication regions. With the available data, we can only speculate on what the repeats do. They may serve as recognition sites for either the membrane or for a protein necessary for replication. Alternatively, they may code for a small repressor RNA as in the case of ColE1 and RI plasmids. A more surprising answer is of course possible.

I would like to draw your attention to F plasmid again. As I mentioned earlier the incD region is common to other plasmids in FI group and is the only region that expresses FI incompatibility. Why do the other inc genes not express incompatibility? DNA heteroduplex mapping using electron microscopy showed that the region containing these genes does not have good homology with DNA of other FI group

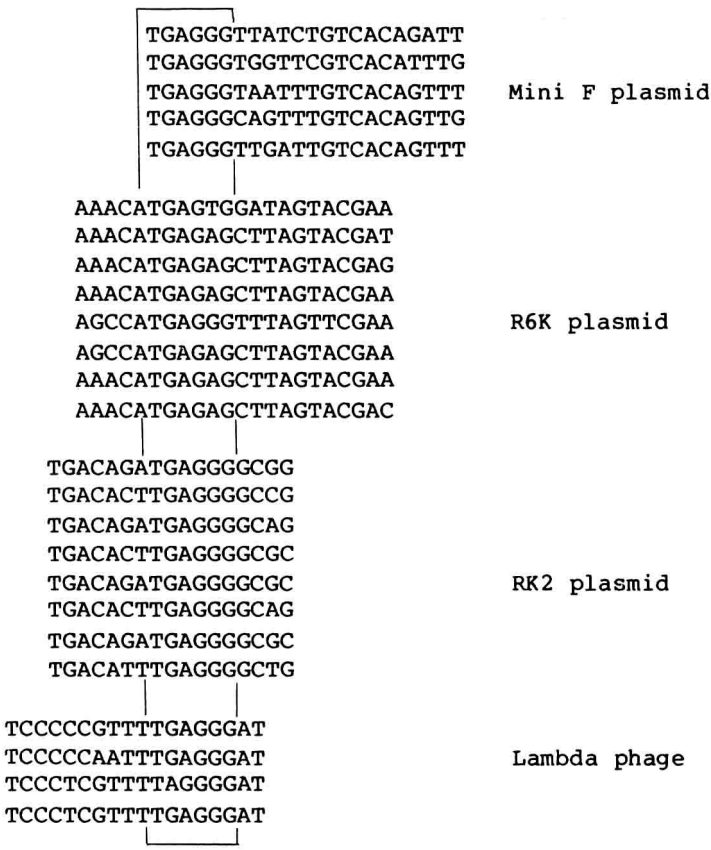

Figure 1. Families of direct repeat units aligned by a box to show the presence of a 6 bp homologous sequence.

plasmids (6). It was interesting to find out whether repeats similar to those in incB and incC were present in other plasmids in the FI group. Southern blot hybridization have shown that all of the four plasmids tested have sequences rather similar, but not identical with the incC repeats (A. Tolun, unpublished observations). Therefore, the overall replication models of the FI plasmids are most probably similar, even though the incB and the incC regions have not been conserved as well as the incD region during the course of evolution. Studies of DNA homology have thus confirmed the classification of plasmids by incompatibility.

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A TRANSPOSABLE ELEMENT FROM HALOBACTERIUM HALOBIUM WHICH INACTIVATES THE BACTERIORHODOPSIN GENE

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INTRODUCTION

Halobacterium halobium belongs to a class of organisms termed "Archaebacteria" by Woose and his coworkers (1). This bacterium displays both prokaryotic and eukaryotic features and is proposed to constitute a major line of descent besides prokaryotes and eukaryotes. One interesting feature of H.halobium is its ability to utilize light energy without involvement of photosynthesis. It is known that light transduction is achieved by means of a membrane protein (bacteriorhodopsin) which catalyzes the light dependent translocation of protons out of cells and hence produces a trans-membrane electrochemical gradient. This gradient is not wasted but is used by cells to synthesize ATP and to drive other transport processes (2).

There are, however, spontaneous mutants (Pum$^-$) of H.halobium which cannot utilize light energy because they lack the protein (bacteriorhodopsin, BR) in their membrane. We have recently cloned and characterized the BR gene from such two mutants and shown (3) that absence of bacteriorhodopsin is due to inactivation of the gene by insertion of a 1.1 Kbp sequence near the NH_2-terminal end. Further characterization of the 1.1 Kbp insertion sequence revealed

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Abbreviations. BR, bacteriorhodopsin ; Pum$^-$, spontaneous mutant of H.halobium which can not synthesize BR ; IS, insertion sequence ISH, insertion sequence from H.halobium ; Kbp, kilo base pairs ; Tn , Transposable element ; RF, replicative form ; ORF, open reading frame.