MOLECULAR ASPECTS OF CHAINIA SP.

A THESIS
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Dedicated

To

My Parents

CONTENTS

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-Vijay M. Chauthaiwale

DECLARATION

This is to certify that the work incorporated in the thesis "Molecular aspects of *Chainia* sp." submitted by Mr. Vijay Murlidhar Chauthaiwale was carried out by the candidate under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

V.V. Deshpande

V.V. Deshpande

Research Guide

ABSTRACT

INTRODUCTION

The genus Chainia was proposed by Thirumalachar in 1955 (1); its main distinguishing feature is the development of vegetative mycelium into sclerotic granules. Older cultures of Chainia produce Streptomyces-like aerial mycelium and sporophores. The taxonomic position of the Chainia genus is a subject of controversy (2,3). While it shows striking similarities with Streptomyces in possessing a high guanine+cytosine (G+C) content in its DNA, strong nucleotide sequence similarity in the DNA of its genome, and a susceptibility to polyvalent Streptomyces phages, certain characters of Chainia, such as abundant formation of sclerotia and the presence of 2,3- diamino propanoic acid in the cell wall, are quite distinctive.

One of the Chainia isolates from our laboratory (Chainia NCL 82-5-1) produces high yields of the commercially important enzymes: xylanase (4) and D-glucose/xylose isomerase (5). Of the two xylanases produced by this strain, one has an exceptionally low molecular weight (6000 daltons) and is cellulase-free. This enzyme is therefore very useful in the paper and pulp industry for the manufacture of hemicellulose-free pulp.

In view of the industrial importance of this

strain, it is proposed to study the various molecular aspects of Chainia by constructing the genomic library of Chainia in E. coli and studying expression of cloned xylanase gene.

The expression of Streptomyces genes in E. coli is a field of great interest in the recent years. Streptomyces genome has very high G+C content and is known to have multiple forms of RNA polymerase holoenzymes (6). In case of cloned Streptomyces genes, recognition of Streptomyces promoters in E. coli is the key step in gene expression. Only a few Streptomyces promoters are found to be expressed in E. coli (7). No data is yet available regarding the expression of Chainia genes in E. coli. Expression of xylanase gene in E. coli, therefore can be used as a model system for the detailed studies on the regulation of expression of Chainia genes in E. coli. In addition, cloning of random DNA fragments showing promoter activity will throw some light on the nature of Chainia promoters.

SUMMARY OF WORK

A: MOLECULAR CLONING OF CHAINIA XYLANASE GENE IN E. coli

1: Construction of Chainia genomic library

The construction of genomic library is the first and most important step in molecular cloning experiments. Once a complete library is available, it can be used for screening and analysis of a number of genes. A standard

approach of shot-gun cloning was used for the construction of genomic library of Chainia in an E. coli vector lambda gt10. It is an insertion vector (8), in which the growth of non-recombinants can be selectively inhibited by plating on E. coli NM514.

The cloning work involved the following steps:

a] Isolation of high molecular weight DNA from Chainia.

b] Standardization of partial digestion conditions using the restriction endonuclease EcoRI.

c] Size fractionation of the partially digested DNA so as to get the fragments in a range of 2-7kbp.

d] Ligation of the size fractionated DNA with EcoRI cut and dephosphorylated lambda gt10 DNA.

e] In vitro packaging of ligation mixture and transfection on permissive (E. coli L87) and non-permissive (E. coli NM514) hosts.

f] Estimation of the titre of the library. The titres of two libraries were 2.48×10^6 and 3.73×10^6 respectively, which represent more than 99% of the Chainia genome.

g] Amplification of library on E. coli NM514 for increasing the proportion of under-represented clones.

h] Confirming the presence of insert by isolation of phage DNA and plaque hybridization with genomic DNA of Chainia.

2: Screening of Genomic Library with Oligonucleotide Probe

Lambda gt10 is not an expression vector. Ideally, a library constructed in this vector is screened using nucleic acid probes. As the complete amino acid sequence of low molecular weight xylanase is available, a 17-mer mixed oligonucleotide probe was synthesized and used for screening. Eleven clones showing hybridization with the probe at medium stringency were propagated and used for screening.

3: Subcloning and screening for xylanase gene expression

The eleven positive clones from the previous step were subcloned in plasmid expression vector pUC8. The recombinants were screened with anti-xylanase antibodies for the expression of xylanase gene (*XylA*). Four recombinants were shown to cross-react with antibodies. One of the four clones showed detectable intracellular xylanase activity. This clone was named as **PVX8**

4: Analysis of PVX8

The expression of cloned *xylA* gene in PVX8 is not induced by IPTG and xylan. It is therefore possible that the gene is regulated by its own promoter. No extracellular activity was detected. It is consistent with the fact that the cloned gene products are generally intracellular in *E. coli*. Even after reorientation of insert (PVX8R), the *xylA* is not induced by IPTG. The

plasmid PVX8 contains an insert of 1.4kbp and has unique sites for HindIII and PstI, two sites for PvuII and no site for BglII and XhoI and BamHI.

5: Genetic transformation of Chainia

In order to develop Chainia as a cloning host, it is important to know whether it possess a restriction system. When Streptomyces lividans broad host range vector pIJ702 was used to transform Chainia protoplasts, the transformation efficiency was very poor (5-10 transformants/μg DNA). However, when pIJ702 isolated from Chainia was used for the transformation, the efficiency is increased by hundred fold, suggesting the presence of restriction system in Chainia. It is also observed that the restriction system in Chainia can be attenuated by heating the protoplasts just prior to transformation, as even a native (unmodofied) pIJ702 gives good transformation efficiency with pre-heated protoplasts.

6: Molecular cloning and expression of Chainia promoters in E. coli.

Sau3AI digested Chainia DNA fragments were size fractionated in a range of 50bp to 500bp and cloned in pJAC4. As pJAC4 has a promoter-less β-lactamase structural gene, it can be used as an indicator gene of a promoter activity (9) Using this approach, a few promoter containing recombinants were isolated and four of them were analyzed in detail. The origin of insert in these

clones was confirmed by colony hybridization using genomic DNA of Chainia as a probe. The four clones (PCP3, PCP4, PCP6 and PCP9) show varying strengths of promoter activity as evident from the different levels of β-lactamase activity and LD_{50} values. While PCP6 is the strongest promoter (LD_{50} = 17μg ampicillin/ml), PCP9 shows weak promoter activity (LD_{50} = 7μg ampicillin/ml).

7: Double helix stability of Streptomyces promoter sequences

The basis of this work in an observation of Margalit et al (10) that in E. coli, a region of 12 nucleotides around -10 has relatively high free energy content which facilitates the localized melting of DNA catalyzed by RNA polymerase. The high free energy content of this region is attributed to the high frequency of dinucleotide pairs viz. AT and TA in and around TATAAT box.

Streptomyces genome has high G+C content. Most of the Streptomyces promoters show very little or no homology with typical prokaryotic consensus sequence. From this point of view, computer analysis of free energy content of the -150 to +50 region of twenty known promoter sequences in Streptomyces was carried out. The following are the significant observations obtained from this study.

1: A region of -9 to +3 shows a pattern of the highest free energy similar to that in E. coli irrespective of

its homology with typical -10 sequence.

2: In case of promoters showing partial homology with that of E. coli, height of the free energy peak at -10 is greater than the corresponding peak for atypical promoters.

3: Contrary to E. coli, there is no preponderance of dinucleotide pairs AT and TA in -10 region. However, the frequency of dinucleotides GG, GC, CG and CC which have relatively poor free energy content is significantly low at -10.

4: In addition to the peak at -10, there are two other regions of high energy at -40 and -80 which are not observed in E. coli.

It is therefore postulated that in spite of high G+C content of Streptomyces genome, though exclusively A+T rich region is absent in these promoters, the high free energy content is maintained by the use of appropriate combination of A or T with G or C.

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Chapter I

Introduction

We may well define science as 'methodical thinking directed toward finding regulative connections between sensual experiences.' It is the aim of science to establish general rules which determine the reciprocal connections of objects and events in time and space. The fact that on the basis of such laws we are able to predict the temporal behavior of phenomena in certain domains with great precision and certainty is deeply embedded in the modern man, even though he may have grasped very little of the contents of those laws.

The quest of understanding these "reciprocal connections", as Albert Einstein has pointed out in one of his articles on 'Science and Religion', enables oneself to explain the various phenomena to varying degree of clarity, certainty and accuracy.

The problems in understanding of various phenomena in living organisms are obviously quite acute. Though living body is composed of lifeless molecules, their coordinated interactions, microenvironment in which they interact and several other factors play an important role in making a living body active and functional. The study of these interactions at molecular level is, therefore, a field of great interest and provides a valuable information on the fundamental aspects of molecular organization in living system. This knowledge is greatly beneficial to several fields of human

interests like health management, environmental protection, agriculture, renewable energy sources etc.

Molecular biology and biomedical research have recently experienced a revolutionary change with the development of gene manipulation techniques. The term gene manipulation or genetic engineering can be applied to a variety of *in vivo* genetics and *in vitro* techniques. In United Kingdom the legal definition of gene manipulation is -

The formation of new combinations of heritable material by the insertion of nucleic acid molecules, produced by whatever means outside the cell, into any virus, bacterial plasmid or other vector system so as to allow their incorporation into a host organism in which they don't naturally occur but they are capable of continued propagation. (1).

The recombinant DNA technology is, therefore, an extremely powerful tool for the detailed understanding of molecular mechanisms such as pathogenicity, regulation of gene expression, differentiation etc.

1.1: RECOMBINANT DNA TECHNOLOGY AND BIOTECHNOLOGY

With the help of gene manipulation techniques, foreign nucleic acid fragments can be introduced in the desired host, stably maintained and propagated overcoming the natural barrier of recombination. These fragments can be sequenced and\or used as highly specific probes. In

vitro site directed mutagenesis of these fragments and replacing such modified fragments into the genome results into modified genetic composition of an organism. In addition, it is now possible to induce genetically tailored microorganisms to express large amounts of commercially important proteins.

Several genes from diverse organisms have been cloned in a variety of hosts. Many of these synthesize commercially important enzymes which are utilized in industry as partial or complete substitute for chemical processes. **Xylanase** is one of these enzymes. Xylanases selectively hydrolyze β-1,4- xylosidic linkages of xylan which is a major constituent of hemicellulose.

1.2: XYLAN AND XYLANASES

Among the several renewable sources of energy that are being considered as alternatives to depleting fossil fuels, photosynthesis holds a great promise. Until more direct methods of trapping solar energy are developed, the most useful method available is the synthesis of biomass by plants and its use as a source of fuel and food. Biomass can be converted by microorganisms to methane, protein or glucose which can be used as a raw material for the production of protein, alcohol or other chemicals.

In the naturally occurring plant materials, cellulose and hemicellulose constitute a major group of polysaccharides. Crop residues such as cereal straws,

bagasse, wheat straw and forestry wastes contain approximately 20-25% of hemicellulose (2,3). According to Flickinger (4), pentose from hemicellulose could be an economical source of carbohydrates for conversion to liquid fuel through microbial fermentation.

The hemicellulose is composed of linear branched heteropolymers of D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid. The types of hemicelluloses are often classified according to the sugar residues present (5). Out of them, the major constituent is xylan which is found in large quantity (30%) in annual plants. β-1,4, xylans are mainly found in woody tissues (6). It is a major component of hemicellulose in angiosperm (15-30%) but is less abundant in gymnosperm (7%-12%) (5).

Xylans are characterized by their properties such as insolubility in water but solubility in 10% alkaline solution. The alkaline solutions of xylan show highly negative optical rotation and are unable to reduce Fehling's solution.

β- 1-4 xylans possess varying degree of polymerization with highly branched structure. The heteropolymer sugars other than D-xylose are also present in xylan. The frequency and composition of branches in isolated xylans depend on their sources (7-9) and on the method of isolation. (5,6,10,11). While esparto grass and

tobacco consist of homolxylans (5,10) i.e. xylan with exclusively xylosyl residues, hard wood consists of acetylated xylan (6).

Xylan can be converted to monosaccharides by enzymatic or acid hydrolysis. Acid hydrolysis is faster but is accompanied by the formation of toxic by-products which can hinder its subsequent microbial fermentation (12).

The enzymatic hydrolysis of xylan involves the action of endo β-1-4 xylanase (1,4, β-D- xylan xylanohydrolase EC 3.2.1.8) and B-xylosidase (1,4,-β-D Xylan xylohydrolase EC 3.2.1.37). In general, the endoxylanses attack internal xylosidic linkages of xylan and β-xylosidases release xylose residues from xylooligosaccharides. The other enzyme Exo β (1-4) D xylanase (EC 3.2.1.91) removes single D-xylose units from the nonreducing end of the xylan chain. D-xylanases produced by bacteria and actinomycetes are mainly of endo type. Though occasionally the presence of an exo-type of enzyme in some fungal cultures has been reported, no conclusive data regarding its isolation, purification or characterization is available (13). Table 1.1 describes various xylanase producing organisms.

1.3: MULTIPLICITY OF XYLANASES

Multiple xylanases have been reported in numerous microorganisms (14). Five endoxylanses have been purified

TABLE 1.1

XYLANASE PRODUCING ORGANISMS

Organism	Strain	reference
Bacillus circulans	WL12	67
Alkalophilic *Bacillus*	C 125	68
Bacillus pumilus	IPO	69
Clostridium stercorarium		70
Clostridium thermolacticum	DSM 2911	71,72
Clostridium aerotolerans	X8A62	73
Clostridium thermocellum		74
Clostridium acetobutalicum	P262	75
Cellulomonas sp	NIAB 442	76
Cellulomonas sp		77
Bacteroides xylanolyticus		78
Bacteroides ruminicola	23	79
Bacteroides succinogenes		80

TABLE 1.1 (Contd)

Organism	Strain	reference
Ruminicoccus flavefaciens	17	81
Fibrobactor succinogenes	135	82
Streptomyces exfoliatus	MC 1	83
Streptomyces flavogriseus		84
Streptomyces lividans		85
Streptomyces olivochromgenes		86
Streptomyces cyaneus		87
Streptomyces roseiscleroticus		88
Streptomyces sp.	EC 1	89
	VP5	90
	T7	91
Chainia	NCl 82-5-1	92
Thermomonospora fusca		93

TABLE 1.1 (Contd)

Organism	Strain	reference
Thermomonospora curvata	MT 815	107
Thermoascus aurantiacus	C 436	94
Talaromyces byssochlamydoides	YH 50	95
Trichoderma harzanum		96
Polyporus tulpiferae		97
Auriobasidium pullulans		98
Aspergillus oryzae		12
Cryptococcus albidus		99

from Aspergillus niger 11 (15), and two from A. oryzae (16). In some cases, multiple xylanases are shown to be the products of distinct genes. In cellulomonas fimi, multiple Xylanases are encoded by distinct genes (17). Distinct xylanase genes have been reported in Ruminicoccus flavefaciens (18), Bacillus circulans (19), Clostridium thermocellum (20,21) and in Pseudomonas flurescens subsp celluosa (22). It, therefore, appear that the multiple xylanases as products of distinct genes is quite a common phenomenon in microorganisms.

1.4: XYLANASES IN BIOTECHNOLOGY

The xylanases have several commercial applications, some of which are listed below:

1] Xylanase finds a potential application in paper and pulp industry for selective removal of xylan from pulp. However, an absence of cellulolytic activity is a prerequisite for its utility. Though cellulolytic activity can be selectively inhibited by mercury (23,24), this leads to the problems of costly waste treatment and disposal. Cellulase-free xylanases stable at alkaline pH is an attractive alternative for alkali treatment during pulping of paper.

2] Conversion of xylan to xylose in the wastes of paper-pulp industry and agriculture can be achieved by cellulolytic preparations containing xylanolytic enzymes.

3] For bioconversion processes, maximum utilization of the various polymeric sugars is desirable. Complete

xylanolytic system involves the action of endoxylanases, β-xylosidases and other debranching enzymes. Complete hydrolysis of xylan leads to the formation of xylose which serves as a ready source of food and fuel. Many prokaryotic organisms have an ability to convert xylose into xylulose by specific isomerases. Inside a cell, xylulose can be converted into xylulose-5- phosphate which enters pentose phosphate pathway. Alternatively, xylose can be converted to xylitol which can be used as an artificial sweetener and as a nonfermentable sugar in chewing gums and toothpastes. Xylose can be fermented to ethanol by few pentose fermenting yeasts such as Pichia stipitis, Candida shehatae and Pachesolen tannophilus or by Neurospora crassa (25-28).

Chainia (NCL 82-5-1) is an isolate from our laboratory which produces two xylanases; one of which being exceptionally low molecular weight and cellulase-free, has tremendous potential in paper and pulp industry.

1.5: TAXONOMIC STATUS OF GENUS CHAINIA

Originally, the genus Chainia was proposed by Thirumalachar in 1955 (29). The chief distinguishing character of this genus is the formation of spherical sclerotic granules in large aggregates from the mycelium. In older cultures, the granules become readily separated from vegetative mycelium and on subsequent transfer to

fresh media germinate and develop into new colonies.

The sclerotia of Chainia are clearly ditinguishable from the interwoven mass of undifferentiated hyphae (30). Micromorphology of Chainia differentiation has presented an evidence for the origin of sclerotia in the well defined sclretotic initials which developed further by growth and division in different planes (31).

Lechevalier et al (32) found that the sclreotial hyphae showed numerous lipid inclusions and a deposition of intracellular cement rich in L-2,3 diaminopropionic acid, an amino acid which was not previously found to be associated with microbial structure.

The taxonomic status of Chainia is controversial (33,34). While some of the characters of Chainia are strikingly similar to Streptomyces such as: (i) high guanine plus cytosine content (ii) DNA homology with Streptomyces genome (iii) susceptibility to polyvalent phages and (iv) stable maintenance of a broad-host-range Streptomyces plasmid pIJ702 (35), the morphological and biochemical characters of Chainia such as formation of rich sclerotia and presence of 2-3 diaminopropionic acid in the cell wall are quite distinctive.

According to Goodfellow et al, the ability of sclerotia formation is transient and therefore cannot be used as a distinguishable character of a genus (34).

However, as described above, the sclerotia are well defined bodies and not merely an aggregation of aerial mycelia. The observations in our laboratory have also revealed that the sclerotia formation is a sequential process and not transient as claimed by Goodfellow et al (35). According to Lechevalier et al (32), Chainia should be recognized as a distinct genus until it has been experimentally demonstrated that Streptomycetes can be induced to form true sclerotia.

For any biotechnological exploitation of xylanases, it is essential to have enzyme preparations produced inexpensively in bulk quantities. The production of xylanases can be improved by various ways such as: a) The optimization of fermentation conditions b) Random mutagenesis and screening of hyperproducer mutants and c) Recombinant DNA technology.

Out of these three approaches, gene cloning using recombinant DNA techniques has several advantages. It is the direct approach as it offers an opportunity to produce single xylanolytic enzyme selected for intended applications or research. The analysis of cloned gene can give extensive information on the regulatory aspects. Further site directed mutagenesis can be used to introduce desired changes in the enzyme molecule.

1.6: VECTORS USED FOR MOLECULAR CLONING

Molecular cloning requires the vehicles that

carry the heterogeneous DNA fragments. These vehicles or vectors are autonomously replicating DNA units into which DNA fragments are inserted. Genes taken up by these vectors are multiplied as the vector replicates.

Many cloning vectors have been developed to meet specific requirements. Though the cloning strategies are developed for a variety of host systems, Escherichia coil still remains the host of choice. Many plasmid and bacteriophage vectors have been constructed for using E. coli as a host.

1.6.1: Plasmid Vectors

Plasmid vectors can be classified into -

a] **General purpose cloning vectors:** In these vectors, cloning of foreign DNA fragments selectively inactivates one of the markers (**insertional inactivation**) or derepresses a silent marker (**positive selection**) so as to differentiate the recombinants from native vector phenotype. (e.g. pBR322). (37).

b] **Expression vectors:** In these vectors, DNA to be cloned and expressed is inserted downstream to a strong promoter present in the vector. The expression of the foreign gene is controlled by the vector promoter irrespective of recognition of its own regulatory sequences provided the coding sequence is in frame and in the correct orientation with respect to the vector promoter.(e.g. pUC18) (38).

c] **Promoter and Terminator Probe Vectors** : With the help

of these vectors one can find out whether the regulatory sequences (like promoters or terminators) in the organism under study can be recognized by the host. These vectors possess a structural gene devoid of promoter or terminator sequence. The fragments containing the promoters can be cloned upstream to the silent structural gene and expression can be studied (e. g. pJAC4) (39).

Generally, it is preferred to clone a desired DNA fragment in a high copy number vector as it facilitates the isolation and purification of gene product in large quantities due to high gene dosage effect. However, if a cloned gene product is toxic to the host, it is desirable that the gene would be expressed at the later stages of growth. High copy number promoter probe plasmids are not suitable for studying the regulation of promoters in vivo (40). In addition, plasmid instablility often occurs when strong promoters are inserted in the high copy number plasmids. (41). Several methods have been developed to circumvent these problems e.g. introduction of transcriptional terminators between cloning site and indicator gene so as to reduce the amount of product synthesized by the strong promoter (42); recombination of vector with chromosome (43) and the use of low copy number vectors like pFZY 1 (44).

1.6.2: Bacteriophage Lambda Vectors:

Bacteriophage lambda has been one of the most

studied viruses and has become a basic vector for molecular cloning due to the interesting features of its genetic organization. It is a temperate phage with a genome size of 48,952 bp (45) and can have two alternate modes of life cycle. When lambda DNA enters a host cell, it can start its replication resulting in the multiplication of phage particles and subsequent lysis of the host cell (**Lytic cycle**) or it can opt for a dormant phase by integrating into the host chromosome resulting in a prophage stage (**Lysogeny**).

Phage lambda vectors have many useful features such as:

a] Acceptance by phage of large DNA fragments increasing the chances of a single clone containing a DNA sequence corresponding to a complete gene.

b] Development and ability of refined techniques aimed at minimizing the problems of background due to non-recombinants.

c] High packaging efficiency.

d] Easy transfection methods.

e] The possibility of screening several thousand clones at a time from single petridish.

f] Easy storage of recombinants.

Lambda vectors are broadly classified into two types.

a) **Replacement vectors:**

On the basis of the well established fact that lambda cannot accommodate DNA molecules smaller than 38

kb or greater than 53 kb (46), not more than 5 kb additional DNA can be inserted into it. However, many of the genes involved in recombination and lysogenization are not essential for phage multiplication which can be deleted (47) and replaced by a foreign DNA. Such vectors in which the non essential DNA in lambda genome is physically eliminated are called as **Replacement Vectors**. In case of these vectors, as the deletion of stuffer fragment results into inefficient packaging, an insertion of foreign DNA constitutes a positive selection for recombinant plaques.

A wide range of lambda replacement vectors have been constructed having different insert capacities and cloning sites. Among them, charon series of vectors are the most versatile. The latest derivatives of charon viz charon 32-40 have many additional advantages (48,49) such as:

1] ability to propagate on $recA^-$ hosts, 2] presence of multiple cloning sites to facilitate cloning, (Charon 36-40 have polylinker consisting of 16 cloning sites), 3] presence of a polystuffer in charon 38-40 to enable easy removal of stuffer and convenient system of detection if the stuffer is inserted back into recombinants, 4] high insert capacity (up to 24kb in Charon 38-40).

Insertion Vectors:

Taking advantage of maximum packagable size (105%

of the wild type lambda genome), instead of removing the central stuffer fragment, an additional fragment is inserted in many vectors. Such vectors are termed as **Insertion Vectors**. Though the insert capacity of these vectors is small as compared to replacement vectors, they are advantageous for cloning of cDNA or small genomic DNA fragments. (e.g. λgt11) (50).

1.6.3: M13 Vectors

Filamentous phages such as M13 are not lytic. They co-exist with the infected cells for several generations and are convenient for cloning genes which produce toxic products. They infect cells via F pilli and the first mature phage appears within 15 minutes.

Phage M13 is widely used in nucleotide sequencing and site directed mutagenesis because its genome can exist either in a single stranded form inside a phage coat or as a double stranded replicative form inside the infected cell. During replication, only the plus strand of the replicative form is selectively packaged by the phage proteins. The replicative form being covalently closed circular (CCC) molecule, can be used as a plasmid vector for usual transformation procedures. The single stranded DNA isolated from phage particles can be used as a template for nucleotide sequencing and for site directed mutagenesis. The vectors derived from M13 viz. M13mp18 and M13 mp19 have the same polylinker as that of

pUC18 and pUC19 respectively (38). The DNA fragments having non-complementary ends can be directionally cloned in this pair of vectors and both the strands of DNA can be sequenced independently.

1.7: MOLECULAR CLONING OF XYLANASE GENES

A number of xylanase genes have been cloned and expressed in heterologous hosts. Though most of these genes are cloned in E. coli, there are some reports regarding their cloning and expression in Bacillus subtilis (51), Streptomyces lividans (52), Lactobacillus plantarum (53) or Saccharomyces cerevaciae (54). Table 1.2 summarizes the sources of cloned xylanase genes.

A variety of vectors have been used for cloning of xylanase genes in E. coli. Plasmid vector pBR322 has been used to clone xylanases gene/s from Bacteroides succinogenes (55), Bacillus pumilus (56), B. polymyxa (57), alkalophilic Bacillus sp strain C-125 (58) and alkalophilic Aeromonas sp No 212 (59).

Taking into consideration the possibility of lack of expression of heterologous genes in E. coli, expression vectors such as pUC series have been used for cloning of xylanase genes from Bacteroides ruminicola 23 (60) Bacteroides ruminicola D31d (61), Ruminicoccus flavefaciens 17 (18), Clostridium thermocellum (62); Bacillus circulans (19); Cryptococcus albidus (63).

There are a few reports of using lambda derived vectors for the cloning of xylanase genes. For example,

TABLE 1.2

LIST OF CLONED XYLANASE GENES

Source	Host	Vector	Reference
Ruminicoccus flavefaciens	*E. coli*	pUC13	18
Clostridium thermocellum	"	pUC8	20,21,62
Clostridium thermocellum	*Lactobacillus plantarum*	pWP37	53
Clostridium acetobutalicum P262	"	pEcoR251	100
Cellulomonas fimi	"	ZAP	17
Cellulomonas sp	"	pUC8	66
Bacteriodes ruminicola 23	"	pUC18	60
Bacteriodes ruminicola D31d	"	pUC18	61
Bacteriodes succinogenes	"	pBR322, pUC8	55
Thermomonospora fusca	"	λgtWES. λB	64
Bacillus pumilus IPO	*S. cerevaciae*	pYPHO5	54
"	*E.coli*	pBR322	56
"	*B. subtilis*	pUB110	51

TABLE 1.2 (Contd)

Source	Host	Vector	Ref
Bacillus circulans	*E. coli*	pUC19	19,101
Bacillus polymyxa	"	pBR322	57
Alkalophilic *Bacillus* C-125	"	pBR322	58,102
Streptomyces lividans	*S. lividans*	pIJ702	52
Streptomyces cyaneus	*E. coli*	gt11	103
Streptomyces sp No 36a	*S. lividans*	pIJ702	104
"	*S. kasugansis* G3	pSK2	104
Psudomonas flurescens subsp. *cellulsa*	*E.coli*	47.1	22
Cryptococcus albus	"	pUC13	63
Caldocellum sacchorolyticum	*E. coli*	pBR322	105,106
Alkalophilic *Aeromonas* No 212	"	pBR322	59
Fibrobactor succinogenes 135	"	λgt WESλB	108

in case of Thermomonospora fusca λgtWES. λB is used (64), while in case of Ruminicoccus flavefaciens 17, λEMBL 3 is used.

The most widely used methods for the screening are based on the ability of recombinants to express xylanase gene. Ruminicoccus flavefaciens 17 was screened using clearance of xylan on plates which was subsequently stained with congo red (18,65). Ability of xylanase synthesizing E. coli recombinants to utilize xylan as a sole source of carbon was exploited to screen xylanase gene from cellulomonas sp. (66). The availability of chromogenic substrate for xylanase further facilitates screening. For example xylanase gene from Bacteroides ruminicola 23 library was screened using RBB-xylan (60). On the other hand, a genomic library of Bacillus pumilus using pBR322 was first screened for β-galactosodase using PNPX as substrate and the same clone is found to carry the xylanase gene (56).

1.8: GENETIC TRANSFORMATION OF CHAINIA

Though E. coli has been used as a cloning host, it will be desirable to clone the genes of interest in the same organism from which it is isolated. As shown in case of S. lividans, self cloning of xylanase gene results in hperproduction of the enzyme (52). In view of developing Chainia as a cloning host, it is important to standardize genetic transformation of Chainia by plasmid

vectors and to study whether Chainia possesses restriction- modification system. Recently, it has been shown that, Streptomyces broad host range vector pIJ702 can be introduced in various Streptomyces species and related genera (109,110). However, no such information was available for Chainia.

1.9: PROMOTER SEQUENCES IN STREPTOMYCES AND CHAINIA

In the recent past, a lot of information is available on the gene regulation in Streptomyces. In summary, Streptomyces has at least seven sigma factors, one of which is involved in differentiation (111). The Streptomyces promoters can be broadly classified into two types -

a] Showing homology with prokaryotic consensus sequences (**Typical Promoters**) and

b] Showing very little or no homology with prokaryotic consensus sequences (**Atypical Promoters**).

Jaurin and Cohen have shown the presence of A+T rich promoters in S. lividans which are recognized by E. coli RNA polymerase (112). To ascertain the presence of similar phenomenon in Chainia, we have isolated few Chainia promoters which are active in E. coli.

The interaction of promoter sequences with RNA polymerase involves localized unwinding of -10 region. Margalit et al have shown that the localized melting at -10 is facilitated by high free energy contents in that

region which is maintained by high frequency of AT and TA dinucleotides at -10 i.e. in TATAAT box (113). In view of high G+C content of Streptomyces genome and poor homology with TATAAT box at -10, it will be interesting to carry out similar analysis in case of Streptomyces promoters.

1.10: PLAN OF THESIS

In the present work, various molecular aspects of Chainia have been studied with special reference to the cloning and expression of Chainia xylanase gene in E. coli. In addition, it gives an account on the presence of heat attenuable restriction system in Chainia and cloning and expression of Chainia promoters in E. coli. The thesis has been organized into five chapters as follows:

CHAPTER I : INTRODUCTION

CHAPTER II : Molecular Cloning and Expression of Chainia Xylanase Gene in E. coli.
The following are the salient features of this chapter:

1: Construction of Chainia genomic library in coliphage lambda derived vector lambda gt10.
2: Screening of library for putative xylanase gene with oligonucleotide probe.
3: Subcloning of the putative xylanase clones in pUC8 and screening for clone/s expressing

xylanase gene.

4: The detailed analysis of one of the xylanase expressing clones viz. PVX8.

CHAPTER III : Genetic Transformation of *Chainia*: Presence of Heat Attenuable Restriction System.

CHAPTER IV : Molecular Cloning and Expression of *Chainia* promoters in *E. coli.*

CHAPTER V : Double helix stability of *Streptomyces* Promoter Sequences.

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Chapter II

Molecular Cloning And Expression Of *Chainia* Xylanase Gene In *Escherichia Coli*

ABSTRACT

A complete genomic library of Chainia was constructed in coliphage lambda vector gt10 and screened for xylanase gene/s using oligonucleotide probe corresponding to a six amino acid sequence of low molecular weight Chainia xylanase. Inserts from eleven putative clones, showing hybridization with the oligonucleotide probe at medium stringency, were subcloned in pUC8. The recombinants in pUC8 were screened for xylanase gene expression using anti-low molecular weight xylanase antibodies. One of the clones showing detectable xylanase activity was named as PVX8. The plasmid pVX8 has an insert of 1.4kbp size with single site for HindIII, PstI and two sites for PvuII. The xylanase activity of PVX8 is not induced by IPTG or xylan. The orientation of insert in pVX8 with respect to lacO/P was reversed by directional cloning of BamHI-EcoRI fragment of insert into pUC9. The resultant recombinant was termed as PVX8R. However, reorientation has no effect on levels of xylanase activity suggesting that the weak expression of indigenous promoter from Chainia is responsible for the xylanase activity in both the clones.

INTRODUCTION

As a result of extensive screening of actinomycetes and Bacillus cultures, several xylanase producing organisms have been isolated in our laboratory. They include Chainia (1), thermotolerant Streptomyces T7(2,3), alkalophilic Streptomyces VP5 (4) and alkalophilic and thermophilic (AT) Bacillus (5). The xylanases from these isolates have been well characterized.

There are several other Streptomyces species known to produce xylanases (Table 1.1). However very few Streptomyces xylanase gene/s have been cloned. Mondou et al have cloned xylanase gene of S. lividans 1326 by functional complimentation of xylanase -ve mutant of S. lividans using pIJ702 (6,7). The cloned gene is xylan inducible and the enzyme levels are 60 times higher than those of wild type.

Similarly, xylanase gene from Streptomyces sp. No 36a has been cloned in S. lividans using pIJ702 (8) Seventy three fold increase in the enzyme levels as compared to donor strain has been observed in the recombinant. When the cloned xylanase gene fragment was transferred into pSK2 series of plasmids and introduced in S. kasugaensis G3, one of the clones showed 47 times increase in the enzyme activity.

Recently, S. cyanus genomic library in lambda

gt11 was screened for xylanase gene on RBB xylan plates. When the recombinants showing clearance were subcloned in pUC8, some of them showed xylanase activity (9).

As described earlier, Chainia (NCL 82-5-1) secretes two distinct xylanases of mol wt 5,500 daltons and 20,000 daltons respectively. Out of them, low molecular weight xylanase has been extensively characterized and sequenced (10). It is a single polypeptide and is free from cellulase and β-xylosidase activity. On wheat bran-yeast extract medium, xylanase activity up to 11U/ml of culture broth was produced in 120h at 28-30°C. The recent studies have shown the involvement of tryptophan and cysteine in the active site of the enzyme (11).

Due to its low molecular weight and cellulase free xylanase activity, the enzyme is commercially important in paper and pulp industry for replacing alkali treatment during pulping. Its low molecular weight makes it an ideal candidate for studying structure-function relationship of xylanases. Molecular cloning of the xylanase gene from this strain will be of great utility in this respect. There are several other ways in which the cloned gene can be studied and manipulated. From these points of view, it is proposed to construct the genomic library of Chainia and study the expression of xylanase clone in E. coli. This chapter describes

i] Construction of Chainia genomic library in E. coli phage vector lambda gt10. ii] its screening with the oligonucleotide probe iii] subcloning of putative xylanase gene in pUC8 iv] studies on the expression of cloned xylanase gene in E. coli and v] restriction mapping of pVX8.

MATERIALS AND METHODS

2.1 CHEMICALS, MATERIALS AND ENZYMES

All the chemicals used throughout the work were of analytical reagent (AR) grade, and were obtained from Glaxo, Sisco or E. Merck, India or British Drug House (BDH), U.K. Tris [Tris (hydroxymethyl) amino methane], SDS (sodium dodecyl sulphate) agarose, ampicillin (sodium salt), PEG 8000 (Polyethylene glycol MW.8000, molecular biology grade), X-gal, IPTG, Xylan (Oat spelt) were obtained from Sigma Chemical Co. U.S.A.

Molecular weight markers viz lambda DNA digested with HindIII and ϕX 174 RF DNA digested with HaeIII were obtained from Boehringer Mannheim GmbH, Germany and BRL, U.S.A. respectively.

Media components viz. tryptone, yeast extract, malt extract and bactoagar were from Difco Laboratories, U.S.A.

All the restriction endonucleases used for digestions of DNA were from Amersham (UK), Boehringer Mannheim (Germany), New England Biolabs (NEB), (U.S.A.), and Pharmacia (Sweden).

Enzymes like DNase I, RNase A (free of DNase activity) Proteinase K were from Sigma chemical co. USA. Polynucleotide Kinase, T4 DNA ligase and calf intestinal phosphatase were from Boehringer Mannheim (Germany). Hybond-N (Amersham U.K.) transfer membranes were used for

Southern blotting of DNA, plaque and colony blotting. Nitrocellulose membrane used for immunoscreening was obtained from Advanced Microdevices, Ambala, India. Elutip-D columns for the purification of DNA were obtained from Schleicher and Shuell.

EcoRI cut and dephosphorylated gt10 arms and *in vitro* packaging extracts were obtained from lambda gt10 cloning kit (Amersham, UK). pUC8 and pUC9 were obtained from Pharmacia, Sweden.

Radiolabelled alpha ^{32}P dCTP or alpha ^{32}P dATP or gamma ^{32}P dATP were from BARC, Bombay. Random primer labelling kit from Boehringer mannheim, Germany was used.

Black and white ORWO photographic film (125 ASA) and X-ray films (Kodak, Fuji) were used for gel photography and autoradiography respectively. Commercially prepared developer (IPC 163) and fixer were used for developing the X-ray films.

2. 2: *E.coli* HOSTS AND VECTOR SYSTEMS USED FOR CLONING

Chainia genomic library was constructed in lambda insertion vector gt10 (12). Subcloning of xylanase gene was carried out using plasmid vector pUC8 (13). The details of genotypes of hosts used, markers, size of vectors and restriction enzyme sites in the vectors are described in Table 2.1 and Table 2.2 respectively.

2.3: MAINTENANCE OF CULTURES AND PHAGE STOCKS

The working stock of *E. coli* cultures was

TABLE 2.1

GENOTYPES OF THE HOSTS

E. coli Strain	Genotype
L87	[*hsd* R ($r_k^- m_k^+$), *trp r*, *met* D, *Sup*E, *Sup*F, Ton A
NM 514	[*hsd* R 514 ($r_k^- m_k^-$), Arg H, gal E, gal X, Str A, lycB7, (hfl^+)]
JM 105	F' *tra*D36 *lacI*^{q} (*lacZ*)M15 *pro* *AB*/*thi* *rpsL* (str^r) *endA* *sbcB15* *(sbcC)* *hsdR4* ($r_k^- m_k^+$) *(lac-proAB)*

TABLE 2.2

PROPERTIES OF THE VECTORS

Name	Size(kb)	Arm Lengths	CloningSite/s
Lambda gt10	43.34	32.71 10.59	EcoRI
pUC8	2.67	----	EcoRI, BamHI, PstI, HindIII, SalI,

stored on agar plates containing appropriate antibiotic, if any, at 4°C for short term while for the long term storage, 15% glycerol suspensions of the liquid cultures were kept frozen at -70°C.

Chainia (NCL-82-5-1) (1) was maintained on MGYP slants and subcultured every three months.

All the lysates of bacteriophage vectors, amplified library and recombinant clones were stored as 1% chloroform stocks at 4°C. For the storage of master stocks, dimethylsulfoxide (DMSO) was added to the bacteriophage lysates to the final concentration of 7% v/v, mixed gently, and stored at -70°C for long term storage.

2.4: MEDIA, BUFFERS AND SOLUTIONS USED

1: **Luria Broth (LB)**

Tryptone : 1%

Yeast extract : 0.5%

NaCl : 1%

pH : 7.2-7.4

2: **MGYP medium**

Peptone : 0.5%

Glucose : 1%

Yeast extract : 0.3%

Malt extract : 0.3%

3: **TNE Buffer**

10 mM Tris.Cl (pH 8.0)

100 mM NaCl

1 mM EDTA (pH 8.0)

4: **TAE Buffer**

0.04 M Tris acetate

1 mM EDTA

5: **GET Buffer**

50 mM Glucose

10 mM EDTA

25 mM Tris.Cl

6: **20 X SSC**

3 M NaCl

0.3 M Tri Sodium citrate

7: **SM Buffer (per litre)**

NaCl : 5.8g

$MgSO_4.7H_2O$: 2g

Tris base : 6.05g

2% geletin : 5ml

pH 7.5 with HCl

8: **10 X PBS**

NaCl : 1.5M

Sodium Phosphate : 0.1M

pH 7.5

9: **PEG/NaCl**

Polyethylene Glycol : 20g

NaCl : 11.7g

The contents were dissolved in 75 ml of SM buffer and

volume was adjusted to 100ml.

10: **50 X DENHARDT'S SOLUTION**

1% (w/v) BSA

1% (w/v) Ficoll

1% (w/v) Polyvinylpyrolidone

All the media were prepared in single distilled water and were autoclaved at 15 psi (pounds per square inch) for 20 min.

For the preparation of solid media agar (2%) was added to the medium before autoclaving and plates were poured after the mixture was cooled to 45°C. For the top agarose, 0.7% agarose was included in the broth.

Heat labile components like antibiotics, IPTG, maltose were filter sterilized through autoclaved millipore (0.45μm) membranes and added freshly to cooled media (45°C).

Ampicillin : Ampicillin (sodium salt) was dissolved at a concentration of 50 mg/ml in sterile autoclaved double distilled water. Effective concentration of 50 ug/ml was used in the medium.

X-gal : (5 bromo-4 chloro 3-indolyl beta D-galactoside) was dissolved in dimethyl formamide at 2% concentration.

IPTG : (Isopropyl thiogalactoside) 10μl of 100mM IPTG and 40μl of 2% X-gal was used per LB plate of 85mm diameter.

2.5: ISOLATION OF Chainia GENOMIC DNA

The genomic DNA of Chainia was isolated according to Hopwood et al (14) with slight modifications. Chainia (NCL 82-5-1) was inoculated from MGYP slant into 50ml MGYP broth in 250ml flask with steel spring and allowed to grow for 36h at 28°C with shaking at 220rpm. The cells were centrifuged at 5000rpm for 15min. 500mg (wet weight) cells were resuspended in 5ml GET buffer containing 2mg/ml lysozyme and incubated for 30min at 37°C. 1.2ml of 0.5M EDTA and 130µl of pronase solution (200µg/ml) were added and kept for 5min at room temperature. 1.4ml of SDS (10%) was added, mixed gently and incubated at 37°C for 2h. When the suspension was clear, equilibrated phenol (6ml) was added, mixed thoroughly followed by the addition of 6ml chloroform. It was then centrifuged at 10,000 rpm for 15min. The aqueous layer was treated again with phenol+chloroform followed by two washes of chloroform. The aqueous layer was then treated with RNase (final concentration 50µg/ml) at 37°C for 30 min. The DNA was precipitated by the addition of 0.1 volume of 3M sodium acetate (pH5.2) and two volumes of ethanol. The spool of DNA was washed with 70% ethanol and dissolved in 1ml TE buffer. 2µl of aliquot from the preparation was loaded on 0.7% agarose gel.

2.5.1: Quantitation of DNA

Each DNA preparation was analyzed for protein and polysaccharide contamination. Absorption spectra of DNA

was recorded in the range of 220-320 nm on a Shimadzu double beam spectrophotometer, model uv 210-A. Only those preparations which exhibited an absorbance ratio A_{280}/A_{260} = 0.45 - 0.55 and A_{230}/A_{260} = 0.45 and A_{300} < 0.05 were used. The equation used for the quantitation of DNA was as follows:

Absorbance of 1 at 260nm = 50μg DNA/ml.

2.6:ISOLATION OF BACTERIOPHAGE LAMBDA DNA

The DNA from lambda gt10 clones was isolated using liquid lysis method (15) with slight modifications.

2.6.1: Preparation of plating cells

The single colony of host culture (E. coli L87 for lambda gt10 and E. coli NM514 for gt10 recombinants) was inoculated in 2ml of LB broth and incubated O/N at 37°C with vigorous shaking. On the following day, 100μl of O/N culture was inoculated in 25ml LB broth containing maltose (4mg/ml) and incubated until the A_{660} reached 0.5. The cells were pelleted by centrifugation at 5,000 rpm for 15min at 4°C. The pellet was resuspended in 12.5 ml of chilled 10mM $MgSO_4$ and stored at 4°C.

2.6.2: Transfection and Lysis

500μl of plating cells were inoculated with a single plaque or approximately 10^7 pfu and incubated for 20min at 37°C. 5ml of LB broth and 5μl of $CaCl_2$ (0.5M) were added and incubated at 37°C for 7 to 8h with vigorous shaking. When the lysis was visualized, 50μl

chloroform was added and kept on shaker for 10min. The lysate was centrifuged for 10min at 5,000 rpm to remove cell debris. The clear lysate was treated with DNaseI and RNase (1μg/ml each) for 30min at 37°C. 5ml of PEG/NaCl solution was added and the solution was kept on ice for 1h and centrifuged at 10,000 rpm for 20min. The solution was drained out carefully to remove PEG. The phage pellet was resuspended in 500μl SM buffer and was briefly centrifuged to remove undissolved particles. It was then treated with 500μl of chloroform to remove the traces of PEG.

2.6.3: Isolation of phage DNA

The 500μl phage suspension in the previous step was treated with 5μl of EDTA (0.5M) and 50μl SDS (10%) and incubated at 65°C for 20min. It was then treated with phenol followed by phenol/chloroform and two washes of chloroform. The DNA was precipitated with equal volume of isopropanol, kept at room temperature for 30min, centrifuged, dried and dissolved in 50μl TE.

2.7:ISOLATION OF PLASMID DNA

The plasmid DNA was isolated according to Kieser with slight modifications (16).

2.7.1: Minipreparations

Two ml of LB with ampicillin (50μg/ml) was inoculated with the culture and grown overnight at 37°C on an orbital shaker. 1ml of overnight grown culture was

centrifuged in the eppendorf tube for 5min. The supernatent was discarded and the cell pellet was resuspended in 100μl GET buffer. 200μl of SDS (1.0%)+NaOH (0.2N) was added and mixed thoroughly. The clear suspension was kept for 5min at room temperature and 150μl of chilled potassium acetate was added. After vortexing briefly, the tubes were kept on ice for 10min and centrifuged for 5min. To the supernatant liquid, 450μl of phenol/chloroform was added, centrifuged and the aqueous layer was treated once with chloroform. The nucleic acids were precipitated by adding 400μl isopropanol and kept at room temperature for 30min. After centrifugation, the pellet was dissolved in 50μl TE, treated with RNase for 30min at 37°C. 20μl from this solution was restricted with appropriate enzyme and visualized on 1% agarose gel.

2.7.2: Large scale plasmid preparation

500μl of overnight grown culture was inoculated into 100ml of LB with ampicillin and incubated further for approx. 16h. The cells were pelleted by centrifugation, resuspended in 10ml GET and treated with 20 ml of 1%SDS/0.2N NaOH. The contents were mixed thoroughly and the tube was incubated at 55°C for 20min. To the clear solution, 15ml of potassium acetate was added, mixed and kept on ice for 30min. After centrifugation, the supernatant liquid was treated with phenol/chloroform followed by a chloroform wash. 35 ml of

isopropanol was added to the aqueous layer, mixed and after incubation for 30min it was centrifuged at 10,000 rpm for 20min. The pellet was washed with 70% ethanol, dried and dissolved in 500μl TE. It was then treated with RNase for 30min, treated with phenol/chloroform and chloroform and precipitated with 50μl 3M sodium acetate and 1ml of ethanol. After overnight incubation at -20°C, the DNA was pelleted by centrifugation, vacuum dried and dissolved in 200μl TE.

2.8: TRANSFORMATION OF E. coli WITH PLASMID DNA

Plasmid molecules can be introduced in a suitable E. coli host by transformation of the competent cells. Though a variety of transformation protocols are available, the following protocol was used which is a slight modification of Maniatis et al (15).

A single colony was inoculated into 5ml LB broth and kept at 37°C O/N with shaking. 100μl of O/N grown culture was inoculated in 25ml LB broth and incubated at 37°C with vigorous shaking till the A_{600} of the culture is 0.5. The culture was chilled on ice for 10 min and centrifuged at 5,000rpm for 10 min at 4°C. The supernatant liquid was discarded and the pellet was resuspended in 12.5 ml 100mM chilled $CaCl_2$/10mM Tris HCl pH8 and kept on ice for 30min. The cells were again pelleted by centrifugation as above and resuspended in 1ml of $CaCl_2$ solution and divided into aliquots of 250μl.

Upto 40ng of supercoiled plasmid DNA was added to each aliquot and incubated on ice for 30min followed by the heat shock at 42°C for 2min. The tubes were transferred immediately to ice for 2min and incubated at 37°C for 60 min without shaking after adding 1ml of LB broth. The different aliquots were plated on LB plates containing amipicillin. The transformation efficiency was expressed as the number of transfomants per microgram of DNA.

2.9: AGAROSE GEL ELECTROPHORESIS

2.9.1: Analytical gels

0.7% agarose gels were used for monitoring digestions of genomic and phage DNA while single and double digestions of plasmids were monitored on 1% or 1.2% agarose gels. The gel electrophoresis was carried out in 1X TAE buffer at a constant current of 50 mA for 3-4h using bromophenol blue as a tracking dye.

After electrophoresis the gels were stained in dark with ethidium bromide (100 ng/ml), visualized on a long wavelength (302 nm) UV - transilluminator (UV products, San Gabriels, California USA) and photographed with a 35 mm SLR camera (Minolta x 700 with microphotography and zoom lens system) using a red filter.

2.9.2: LMT agarose gel electrophoresis

Low melting temperature (LMT) agarose was used to elute specific DNA fragments from the gels. LMT agarose

was melted in TAE buffer by heating at 70°C for 30min and cooled to 37°C. After pouring the gel, it was allowed to solidify for 1h at 15°C. After completion of electrophoresis, it was stained with ethidium bromide as above and visualized on U.V. transilluminator. The specific bands were cut with the help of a sharp razor blade and collected in eppendorf tubes. To this approximately 5volumes of TE buffer was added and heated at 65°C for 20min so that the agarose is completely melted. To the molten agarose, equal volume of phenol was added and mixed thoroughly. It was centrifuged in eppendorf centrifuge for 10 min and treated with phenol+chloroform until the interphase was not visible. It was then washed twice with chloroform and DNA was precipitated with 2.5vol of ethanol. The tubes were kept at -70°C overnight and DNA was pelleted by centrifugation. It was then repeatedly washed with 70% ethanol, dried and purified on Elutip-D column as follows:

The DNA sample was dissolved in low salt buffer (0.2M NaCl; 20mM Tris.HCl pH 7.4; 1.0mM EDTA). The column was washed with 3ml of high salt buffer (1.0M NaCl; 20mM Tris.HCl pH 7.4; 1.0mM EDTA) followed by priming of column by two washes of 5ml each by low salt buffer. DNA sample was absorbed on to the primed column by forcing it slowly with the help of a syringe. The column was washed twice with 3ml low salt buffer and DNA was eluted

with 0.4ml of high salt buffer. The DNA was precipitated with two volumes of ethanol, centrifuged and the pellet was washed twice with 70% ethanol to remove the salts. DNA was dissolved in TE.

2.10: RESTRICTION DIGESTIONS

2.10.1: Complete digestions

For all digestions of plasmid DNA with restriction enzymes, minimum 10 U of restriction enzyme per microgram of DNA was used in a reaction volume of 20 µl at 37°C for appropriate time. Either the commercially supplied buffers or the buffers prepared according to Maniatis et al (15) were used. The enzyme digestion was stopped by the addition of EDTA to a final concentration of 10mM.

2.10.2: Partial digestion of Chainia DNA

To generate 2-7kbp DNA fragments, Chainia DNA was partially digested with EcoRI restriction endonuclease. A solution containing 10 µg of genomic DNA with 10 µl high salt buffer was adjusted to a final volume of 100 µl. It was dispensed in eppendorf vials with 20 µl in first vial and 10 µl each in the remaining vials kept on ice. 20 units of EcoRI were added to the first vial and the contents were mixed thoroughly but gently. The tube was microfuged and 10 µl of the sample was transferred to the next vial and the serial transfers were carried out up to the 8th vial. The 9th vial was left as such without any addition. The vials were

briefly microfuged and incubated at 37°C for 1h. The reaction was terminated by chilling to 0°C and adding EDTA to a final concentration of 10mM. The DNA was analyzed by electrophoresis on 1% agarose. The partially digested DNA was extracted with phenol/chloroform and precipitated by addition of 2 volumes of ethanol after adjusting sodium acetate concentration to 0.3 M. After centrifugation the pellet was dissolved in 50 ul TE.

2.10.3: Double Digestions of DNA

For the double digestion, DNA was first digested with the enzyme requiring low salt buffer, followed by incubation with high salt requiring enzyme. Digestions were allowed to proceed at 37°C. The reaction was terminated as described above.

2.10.4: Size Fractionation

The size fractionation of the restricted DNA was carried out by sucrose density gradient centrifugation (14). The sucrose gradient was prepared as follows: 6ml of10% sucrose in TNE buffer was carefully loaded on the top of 6ml of 40% sucrose in TNE buffer. The tube was sealed and rotated slowly to the horizontal position and kept at room temperature for 4h. The tube was then gently turned back to the vertical position and 50μg of DNA digested partially with EcoRI was loaded on the top. It was then centrifuged at 35,000rpm for 16h at 20°C in Beckman SW41 swing-out rotor. After centrifugation,

fractions of 300μl each were collected and 10μl of every alternate fraction was loaded on 0.8% agarose gel. The fractions (5 to 11) ranging from 2kb to 7kb were pooled and precipitated with equal volume of isopropanol.

2.10.5: Dephosphorylation

Linearized plasmid vectors were dephosphorylated with calf intestinal alkaline phosphatase (CIP). DNA was dissolved in 50μl phosphatase buffer (50mM Tris. HCl pH7.5, 1mM EDTA, 10mM $MgSO_4$) and incubated with 1U of enzyme for 1h at 37°C. The phosphatase was removed by the treatment with phenol/chloroform.

The double digested plasmids having non-complementary ends were not dephosphorylated.

2.11: SYNTHESIS OF OLIGONUCLEOTIDE PROBE

An 18-mer oligonucleotide sequence was synthesized on Cruchm Manual synthesizer in collaboration with Dr. Ganesh, N.C.L., Pune. The sequence chosen for this purpose is a stretch of 6 amino acids in low molecular weight xylanase. The amino acid sequence and the corresponding DNA sequence is as follows -

Met - Tyr - Tyr - Ser - Phe - Trp

Codons: 5'AUG - UAU - UAU - UCU - UUU - UGG 3'
C C C C
A
G

DNA sequence of the non-coding strand -

```
5' ATG - TAT - TAT - TCT - TTT - TGG 3'
           C     C     C     C
                       A
                       G
```

2.12: BLOTTING

2.12.1: Southern blotting

The transfer of DNA from agarose gels to Hybond N (Amersham) membranes was carried out according to Maniatis et al (15).

After electrophoresis the gel was soaked in 0.25 N HCl, at room temperature for 10 min for depurination of DNA. The DNA was then denatured by soaking the gel twice in 1.5 M NaCl and 0.5 M NaOH for 30 min at room temperature with gentle shaking. The gel was neutralized by soaking it in 1M Tris.HCl, pH 7.4 and 1.5 M NaCl, 30 min, twice with constant shaking. The capillary blotting of DNA onto the membrane was carried out for 12-15 hr in 20 X SSC, after which the membrane was rinsed in 5 X SSC, air dried and exposed to U. V. irradiation for 5min with DNA side facing the U.V source.

2.12.2: Plaque Blotting

Plaque blotting was carried out as described in Amersham's blotting and Hybridization protocol book.

The plates containing plaques were precooled at 4°C for 1.5 to 2h. Hybond N circles (85mm) were placed on the precooled plates containing plaques for 4-5 min. 3

asymmetric holes were made on the paper through the plate.

Membranes were gently lifted and kept on the Whatman No 3 filter presoaked in denaturation solution (0.5M NaOH, 1.5 M NaCl) for 7 min. This was followed by 3 min neutralization by keeping on the whatman No 3 filter presoaked in neutralizing solution (1.0 M Tris.HCl pH 7.5, 1.5 N NaCl) twice. The blots were briefly washed with 2X SSC and air dried. The blots were prepared in duplicates from each plate. For the second lift again 3 asymmetric duplicate holes other than the 1st lift were made. All the holes were marked on the plate which were later used for aligning the signals on X-ray film with the plates.

2.12.3: Colony Blotting

The LB agar plate containing ampicillin was covered with Hybond N circle of the appropriate diameter. The colonies were patched on the membrane and allowed to grow overnight. The membrane was lifted and treated as in case of plaque blots as described above.

2.13: LABELLING OF DNA

2.13.1: Random priming and labelling

Probes were labelled with alpha ^{32}P dCTP (Specific activity 3000 Ci/m Mole (BARC) by random priming method of Fienberg and Vogelstein (17,18) which is based on the hybridization of a mixture of random hexanucleotides to

the DNA to be labelled, which acts as a primer for synthesis of a second strand of DNA in which ^{32}PdCTP is incorporated. Using this method, it is possible to generate probes of specific activity > 10^8 CPM/ug DNA. The length of DNA fragments to be labelled does not influence the reaction. Since input DNA serves as a template and remains intact during the reaction, it is possible to label minimal amounts of DNA (10 ng) to a high specific activity.

50ng of the probe DNA in a suitable volume of TE was denatured by boiling for 10 min and was immediately chilled on ice. To the denatured DNA following reagents from Boehringer random primer labelling kit were added in series. 2μl reaction buffer containing primer, 1μl each of dATP, dGTP, dTTP, and 5 ul of alpha^{32}P dCTP (specific activity 3000 Ci/m Mole) and 2 ul Klenow fragment (4U). Final volume was made up to 20 μl with sterile water and the reaction was carried out at 37°C for 1 h. The reaction was stopped by adding EDTA to final concentration of 10mM.

2.13.2: Purification of probe

To prevent background or nonspecific hybridization, in radioactive experiments, it is necessary to separate the probe from unincorporated labelled nucleotides. This is achieved by purification of the probe by spun column chromatography (15).

To begin with a 1.5 ml microfuge tube was packed

with 1ml Sephadex G-50 equilibrated with TE. The entire mixture of probe, after the reaction, was pipetted onto this exclusion column. 50 ul of 25 mM EDTA/1% SDS was added directly on top of the column, and after waiting for 30 sec, the column was spun at 1000 rpm for 20-30 seconds in Remi table-top centrifuge. The probe eluted in the volume of about 350-400 ul was collected and used for the hybridization experiment.

2.13.3: End Labelling

Approximately 65pmols of 18mer oligoprobe was end labelled using T4 Kinase and τ^{32}P ATP. The labelled probe was purified by precipitation with ethanol and used for hybridization (19).

2.13.4: Counting of the radioactivity.

The radioactivity of the purified labelled probes was counted in Rack-beta liquid scintillation counter.

2.14: HYBRIDIZATION AND WASHING

Before hybridization with labelled probe, it is essential to block the sites on the membrane that bind single or double stranded DNA nonspecifically giving high background to the autoradiograms. Prehybridization with non specific DNA (such as calf thymus DNA) serves this purpose.

2.14.1: Prehybridization

Southern blots or plaque screens were prehybridized for 3-4 h at 62°C in heat sealed plastic

bags containing the following mixture: 5 X SSC 0.1% SDS, 5 X Denhardt's solution and 100 ug/ml calf thymus DNA. The amount of prehybridization solution added, was 200ul/Cm^2 of the blot.

2.14.2: Hybridization:

After prehybridization, the solution was removed, and replaced by hybridization solution containing the probe. The purified probe was denatured by boiling it together with hybridization solution for 10 min, and immediately added to the blots. Hybridizations were carried out overnight with gentle shaking.

2.14.3: Washing of filters and autoradiography.

After hybridization, the filters were washed for removal of the unbound/nonhybridizing probes using solutions of different stringencies depending on the nature of the probe used for hybridization.

The filters used for hybridization with homologous probe were washed twice with 2 X SSC + 0.1% SDS, each for 10 min at room temperature with gentle shaking. This was then followed by 2 washes at 62°C for 10 min with 1X SSC 0.1%SDS and 0.1X SSC, 0.1% SDS respectively. Moist filters were wrapped in saran wrap and exposed to X-ray film (Kodak) for 12 hr to 4 days at -70°C using vinyl X-ray cassettes with built in Du Pont superfast intensifying screens.

2.15: CONSTRUCTION OF Chainia GENOMIC LIBRARY.

2.15.1: Preparation of target DNA

Isolation of Chainia DNA and its partial digestion with EcoRI were according to the procedures described in sections 2.5 and 2.10.2 respectively. Partially digested Chainia DNA was size fractionated on sucrose density gradient as described in section 2.10.4.

2.15.2: Ligation with the vector

The ligation reactions were carried out as shown in the Table 2.3 and incubated at 14°C for 16h.

2.15.3: In vitro packaging

In vitro packaging extracts A and B were thawed on ice. Extract A (10μl) was added to the ligation mixture followed by addition of extract B (15μl). The tube were centrifuged briefly and incubated at 20°C for 2h. 0.47ml SM buffer was added and stored at 4°C.

2.13.4: Transfection

10μl of packaged phage mixtures from V10, V2 and V3 were added to 90μl SM each and then serially diluted in duplicate upto a dilution of 10^8. The top agar was molten and kept in 45°C water bath. 100μl of suitably diluted phage mixture (Table 2.4) was added to 100μl of freshly prepared E. coli L87 and NM514 plating cells (section 2.6.1) and kept at 37°C for 20 min. This mixture is then added to 4ml of top agar, mixed thoroughly and poured on LB plates. After allowing the soft agar to solidify, the plates were kept O/N at 37°C. The

TABLE 2.3

LIGATIONS OF THE VECTOR ARMS AND THE INSERT DNA

	INSERT DNA	gt10 DNA	gt10 arms	10X Ligase buffer	Water	T4 DNA Ligase
V10:	NIL	5μl (0.5μg)	NIL	1μl	3μl	1μl (2.5U)
V2:	2μl (100ng)	NIL	2μl (1μg)	1μl	4μl	1μl
V3:	4μl (200ng)	NIL	2μl (1μg)	1μl	2μl	1μl

TABLE 2.4

PLATING OF PACKAGED MIXTURE ON DIFFERENT HOSTS

Sample	Dilutions Plated On	
	L87	NM514
V10	10^5, 10^6, 10^7	10^3, 10^4, 10^5
V2	10^4, 10^5, 10^6	10^4, 10^5, 10^6
V3	10^4, 10^5, 10^6	10^4, 10^5, 10^6

packaging efficiency was defined as the number of pfu obtained per microgram of phage DNA on E. coli L87.

2.16: ANALYSIS OF Chainia LIBRARY IN gt10.

After the transfection, the number of plaques at each diltuion on both the hosts was scored and the titre of the original packaged mixture was determined.

2.16.1: Amplification

30μl of V2 and V3 phage mixture was diluted to 10^3 and plated on NM514. The confluent lysis was observed on the following day to which 5ml SM was added and kept overnight at 4°C. On the following day, the lysate along with soft agar was collected, centrifuged and the supernatant liquid was stored at 4°C after addition of 500μl of chloroform. The lysates were labelled as V02 and V03 respectively and their titres were measured after suitable dilution as above.

2.16.2: Plaque blotting and Hybridization

V02 and V03 were suitably diluted so as to get isolated plaques. The plaques were blotted on Hybond N as described in section 2.12.2 and challanged with total genomic DNA of Chainia labelled with ^{32}P dATP. Prehybridization, hybridization and washing were carried out as described in section 2.13 and membranes were exposed to X-ray film for 12 h.

2.16.3: Phage DNA minipreparation

The isolated plaques were picked up with the help of a sterile pasteur pipette and separately suspended in 500µl SM. The suspensions were kept for 2 to 3h at 4°C to allow pahge particles to diffuse into the liquid and used to transfect on NM514 plating cells.The phage DNA was isolated according to section 2.6. The DNA was restricted with EcoRI and the average size of the insert was measured.

2.17: SCREENING OF LIBRARY WITH OLIGOPROBE FOR XYLANASE CLONE/S

The plaque blots representing approx. 10,000 clones were challenged with labelled oligo probe. The hybridization was carried out for 24 h at 45°C in a solution containing 6 X SSC; 0.1% SDS; 5X Denhardt's solution and 100µg/ml denatured calf thymus DNA. The filters were washed three times at room temperature in 6 X SSC; 0.05% SDS for 15 min each followed by a wash by 6 X SSC; 0.05% SDS at 48°C for 30 min. After autoradiography, the filters were rewashed by 3 X SSC; 0.05% SDS at 55°C for 10 min for subsequent exposure.

2.18: SUBCLONING IN pUC8

The clones showing hybridization with the oligoprobe at medium stringency were picked up and propagated by liquid lysis method. The inserts were eluted from LMT agarose and pooled. The pooled

preparation of inserts was ligated with EcoRI cut and dephosphorylated pUC8 and transformed in E. coli JM105 according to section 2.8 The transformation mixture was plated on ampicillin plates containing X-gal and IPTG. The white colonies were selcted and grown on ampicillin plates. The origin of insert was confimed by colony blotting and hybridization with ^{32}P labelled genomic DNA of Chainia.

2.19: IMMUNOSCREENING

The white colonies (i.e. recombinants) obtained after subcloning in pUC8 were screened for xylanase expression as follows (20).

colonies were grown on the LB+ ampicillin plate covered with sterile nitrocellulose membrane. The membrane was then lifted and immersed in PBS buffer containing lysozyme (2mg/ml) and BSA (100μg/ml) and kept overnight. It was then washed twice with PBS buffer (10min each) and then challaged with anti-low molecular weight xylanase antibodies. The membrane was incubated with 1:100 diluted antibodies in PBS buffer containing 100μg/ml BSA for 1 h with gentle shaking. It was washed thrice with PBS (10min each) and challanged with anti-rabbit IgG - peoxidase conjugate (1:2000 diluted in PBS containing 100μg/ml BSA) and kept for 1h. After washing thrice with PBS the bound peroxidase was detected with

0.5% 3,3 diaminobenzidine tetrahydrochloride in 0.5M sodium citrate buffer pH5, containing 0.03% H_2O_2.

2.20: EXPRESSION OF XYLANASE GENE

2.20.1: Preparation of cell extracts

To study the expression of xylanase gene/s in E. coli, the clones showing positive signal after immnoscreening were propagated in 50 ml of LB+ampicillin. The cells were pelleted by centrifugation and broth was stored at -20°C. The cells were washed with 500mM phosphate buffer pH7.2, centrifuged and resuspended in 2ml phosphate buffer pH7.2 containing 200mM guanidine hydrochloride and 0.5% Triton X-100 (21). It was then kept at 4°C for 12-16h. The cells were separated by centrifugation and supernatant was stored at -20°C (Fraction I). The pellet was resuspended in 1ml of phosphate buffer and frozen at -20°C. It was then thawed under running tap watar, centrifuged, and supernatant was named as fraction II.

2.20.2: Estimation of xylanase activity

2g xylan was suspended in 100ml of sodium acetate buffer (50mM) pH5.0 and was stirred for 12-16h. The insoluble fraction (about 50%) was removed by centrifugation and the soluble fraction was used for xylanase assay.

Xylanase was assayed by mixing a 0.5ml aliquot of appropriately diluted enzyme with 0.5ml of 1% xylan and

incubated at 50°C for 30min. The reducing sugar was determined by DNSA method with D-xylose as standard (22). Unit of xylanase was defined as the amount of enzyme which produces 1μmol of xylose per minute from xylan in the described assay cinditions.

2.20.3 : **Immunodiffusion**

Ouchterlony double diffusion was carried out in 1% agarose gel in 1 X PBS at 4°C for 24-48h. Precipitin lines were visualized after drying the gels and staining with 0.25% Coomassie brilliant blue R-250.

2.21: SOUTHERN BLOTTING AND RESTRICTION MAPPING OF pVX8

pVX8 plasmid DNA and genomic DNA of Chainia were digested with different enzymes. The electrophoresis was carried out on two separate gels and the DNA samples was transferred on to Hybond-N as described in section 2.12.1. While the blot of the plasmid DNA was then challanged with lebelled genomic DNA of Chainia, the blot of genomic DNA of Chainia was challanged with EcoRI+BamHI double digested and eluted insert DNA of pVX8. For the restriction mapping, pVX8 plasmid was isolated from 100ml culture as described in section 2.7.2. It was then digested with EcoRI, BamHI, BglII, HindIII, PvuII, XhoI, or PstI. To confirm the actual location of sites, EcoRI+BamHI and BamHI+PvuII digestions were carried out as described in section 2.10.3.

2.22: REORIENTAION OF pVX8 INSERT

pVX8 was digested with EcoRI+BamHI and the insert fragment was eluted from LMT agarose and purified on Elutip-D column as described in section 2.9.2. It was then ligated with ECoRI+BamHI digested pUC9 so as to reverse the orientation of the insert with respect to LacO/P of pUC8. The plasmid DNA isolated from the new recombinant (**PVX8R**) was digested with EcoRI+BamHI to confirm the insert size.

RESULTS

2.23: CONSTRUCTION OF *Chainia* GENOMIC LIBRARY

The main objective of the present work was to construct a genomic library of *Chainia* in *E. coli* and screen it for xylanase clone/s. To construct a library, a standard strategy of shot-gun cloning was adopted in which the partially digested DNA fragments are ligated to the vector molecules and the resultant chimeric molecules are propagated on a suitable host. In the present work, bacteriophage lambda derived insertion vector lambda gt10 was used (Fig 2.1). The results of the work are described in the following sections.

2.13.1: Partial digestion and size fractionation of *Chainia* DNA

Since the insert capacity of lambda gt10 is between 0 to 7.2 kb (12), it was essential to prepare the insert DNA in the range of 2 to 7 kb to avoid the ligation of very low molecular weight DNA fragments with the vector. The standardization of conditions for partial digestion revealed that digestion for 1h with 2.5U of EcoRI/μg DNA was sufficient to generate the DNA fragments in the required range (Fig 2.2). 100μg of *Chainia* DNA was digested with EcoRI. After stopping the reaction with EDTA it was subjected to sucrose density gradient centrifugation so as to eliminate the fragments which lie outside the selected range. Fig 2.3 shows the

FIGURE 2.1

Restriction Map of Lambda gt10

E-EcoRI
B-BamHI
P-PvuII
K-KpnI
Sm-SmaI
H-HindIII

FIG. 2·1

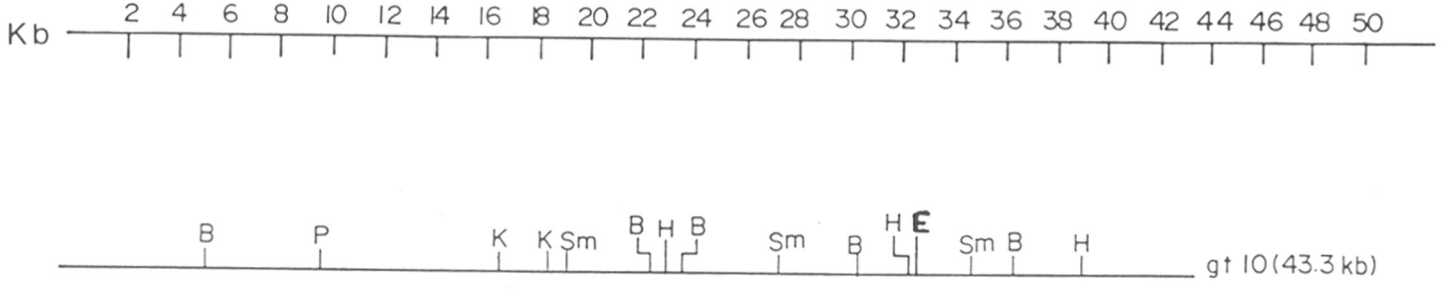

Kb
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
B P K K Sm B H B Sm B H E Sm B H
gt 10 (43.3 kb)

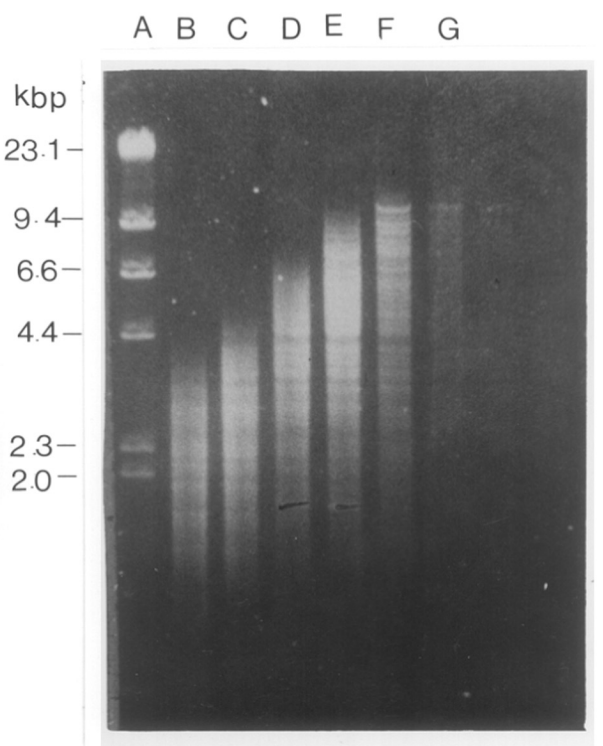
FIGURE 2.2

Standardization of Partial Digestion Conditions

Lane A : Lambda HindIII marker

Lane B to Lane G : *Chainia* DNA digested with decreasing units of EcoRI

FIG. 2.2



electrophoresis of the size fractionated DNA. The fraction number 5 to 11 were pooled, precipitated, dissolved in TE and used as the source of insert DNA.

2.23.2: Ligation, In vitro packaging and Transfection

The size fractionated DNA was ligated with gt10 arms as described in materials and methods and transfected on E. coli L87 and NM514 hosts. Table 2.5 shows the number of pfu obtained on each of the hosts and the in vitro packaging efficiency using gt10 DNA and the ligation mixtures. The packaging efficiency of parental phage DNA is > 10^8 which is comparable to the reported value.

2.24: ANALYSIS OF LIBRARY

2.24.1: Titre of library

E. coli NM 514 allows the lytic growth of only cI^- mutants. As evident from the Table 2.5 , the ratio of pfu of gt10 on L87 to that on NM514 is more than 450:1. This shows that the percentage of the parental phages having cI^- mutation is very low. On the other hand, the similar ratio for the ligation mixture is almost 1:1 indicating that the background of parental phages is extremely low and the growth on NM514 is due to the insertional inactivation of cI repressor gene.

2.24.2: Phage DNA isolation and plaque blotting

The presence of insert was further confirmed by picking the plaques randomly, propagating them on E. coli

FIGURE 2.3

Size Fractionation of EcORI digested Chainia DNA

Lane A to Lane J : Fraction No 5 to 20 loaded alternatively.

Lane K : Lambda HindIII+EcoRI marker.

FIG. 2.3

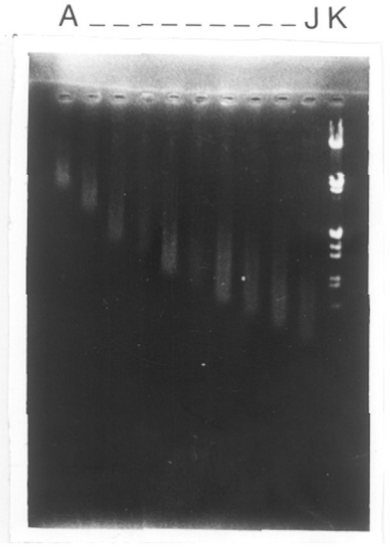


TABLE 2.5

PLAQUE FORMING UNITS ON DIFFERENT HOSTS

Name	pfu on 87	pfu on NM514	Ratio of pfu L87 : NM514	Packaging Efficiency on L87
V10	1.1×10^8	2.3×10^5	478:1	2.2×10^8
V2	2.9×10^6	2.48×10^6	1.16:1	2.9×10^7
V3	4.1×10^6	3.73×10^6	1.09:1	2.05×10^7

NM514 by liquid lysate method followed by phage DNA isolation and restriction with EcoRI. The blots of the plaques grown on E. coli NM514 show intense hybridization signals (Figure 2.4), when challenged with the labelled genomic DNA of Chainia, confirming the origin of inserts. Figure 2.5 shows the presence of insert in several of gt10 recombinants. The average size of the insert was found to be approximately 3.5kbp.

2.24.3: Representation of Chainia genome in the library

Hopwood et al have estimated that with an average insert size of 5kbp, more than 18,000 clones are required to have more than 99% probability of complete library (14). Assuming the genome size of Chainia to be approximately same as that of Streptomyces, (10^4 kbp) (23) and using the formula:

$$N = \frac{\ln (1 - P)}{\ln (1 - f)}$$

where: N = Necessary number of recombinants.

P = The desired probability.

f = The fractional proportion of the genome in a single recombinant.

It was concluded that 2 X 10^4 clones of an average insert size of 3.5kbp are required to have 99% probability of the complete library. As seen from the Table 2.5, both V2 and V3 represent at least 10 times

FIGURE 2.4

Plaque Hybridization of Lambda gt10 Library With Labelled Genomic DNA of Chainia.

FIG. 2.4

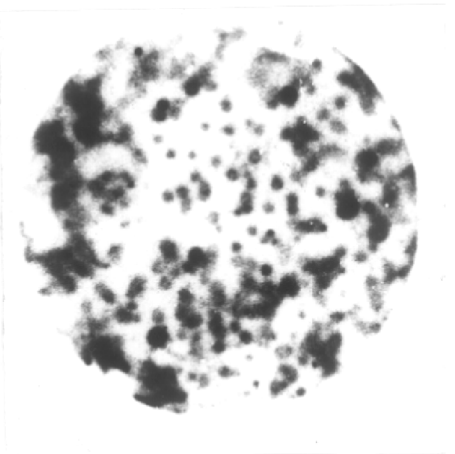


FIGURE 2.5

Gel Electrophoresis of EcoRI Digested DNA of Lambda gt10 Recombinants.

FIGURE 2.5a and 2.5b

Lane B to Lane O : EcoRI digested DNA of gt10 recombinants

Lane A and Lane P : Lambda HindIII marker.

FIG. 2.5 a

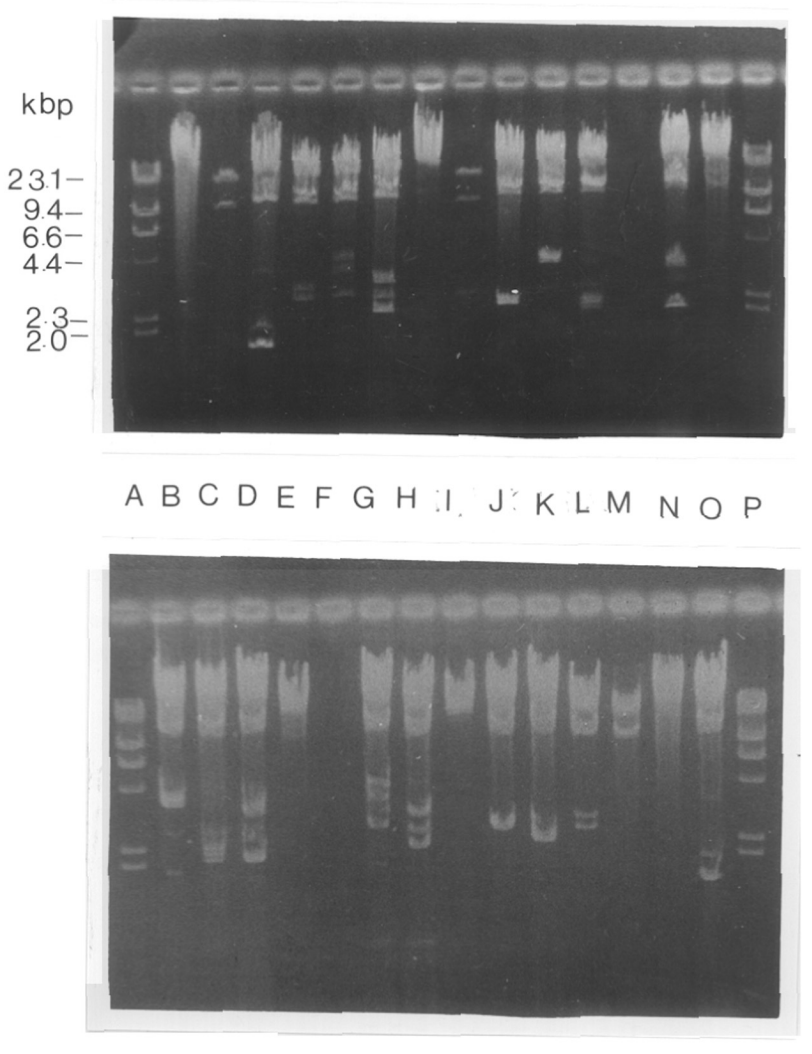


FIG. 2.5 b

more number of clones than those required to constitute 99% of the library.

2.24.4: Amplification

To increase the probability of representation of each clone, both the libraries were amplified by a confluent lysis on agar plates. The pooled phage suspension was collected and stored at 4°C with chloroform and also at -70°C with DMSO. An aliquot of each amplified library was diluted suitably to obtain the exact titres. The titres of the two amplified libraries were 5×10^{10} and 6.9×10^{10} respectively.

2.25: SCREENING OF GENOMIC LIBRARY WITH OLIGONUCLEOTIDE PROBE

A library in lambda gt10 can be easily screened with nucleic acid probes. As the complete amino acid sequence of the low molecular weight xylanase from Chainia is known, a mixed oligonucleotide probe was synthesized to screen the library. A mixed probe, as described in the section 2.11, contains all the possible sequences representing a stretch of six amino acids in the low mol. wt. xylanase.

At a low stringency, the oligo probe showed hybridization with more than 20 plaques (Fig 2.6a). However, the subsequent washing of the filters at medium stringency showed hybridization with eleven clones (Fig 2.6b). These clones were picked up, amplified on E. coli

FIGURE 2.6

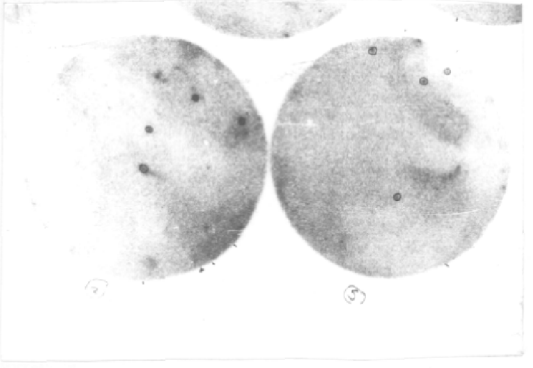
pLAQUE Hybridization of gt10 Library With The End-Labelled Oligonucleotide Probe.

FIGURE 2.6a : Hybridization at low stringency.

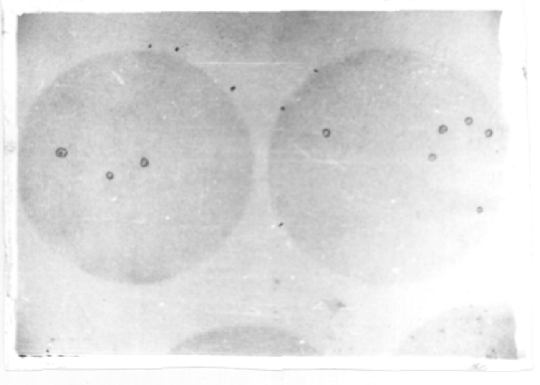
FIGURE 2.6b : Hybridization at medium stringency.

FIG. 2.6

a



b



NM514 and stored at 4°C.

2.26: SUBCLONING IN pUC8

As lambda gt10 is not an expression vector, it was essential to subclone the DNA fragments with putative xylanase gene into a suitable plasmid vector to study its expression. For this purpose, the pooled insert preparation of eleven clones which show hybridization at medium stringency was cloned in pUC8. The origin of insert of the recombinants (i.e. white colonies on X-gal + ampicillin plates) was confirmed by colony hybridization of ^{32}P labelled Chainia DNA (Fig 2.7). The recombinants were then challenged with anti xylanase antibodies and four clones showing cross-reaction (Fig 2.8) were named as PVX1, PVX4, PVX8 and PVX16. All the four were grown in LB broth and cell extracts were prepared as per Section 2.20.1. Both the culture broth and cell extract were estimated for xylanase activity. Only the cell extract from PVX8 showed detectable xylanase activity. Labelled insert of pVX8 showed hybridization with restricted genomic DNA of Chainia (Fig 2.9) and labelled Chainia DNA showed hybridization with the plasmid (Fig 2.10).

2.27: EXPRESSION OF XYLANASE GENE BY PVX8

2.27.1: Xylanase activity

Table 2.6 shows the activity of the cell extracts and the broth of PVX8 culture. Only after the combined

FIGURE 2.7

Colony Hybridization of pUC8 Recombinants With Labelled Genomic DNA of Chainia.

FIG 2·7

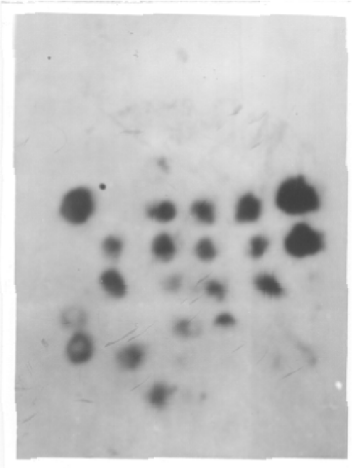


FIGURE 2.8

Immunoscreening of pUC Recombinants for Xylanase Gene.

FIG. 2·8

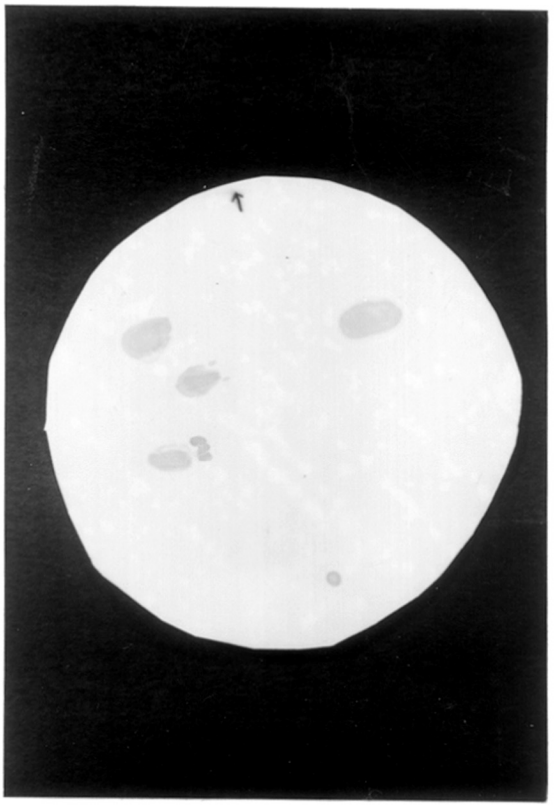


FIGURE 2.9

Southern Hybridization of Restricted *Chainia* DNA With Labelled Insert

Lane A : EcoRI

Lane B : PstI

FIG.2.9

FIGURE 2.10

Southern Hybridization of Restricted pVX8 DNA With Labelled *Chainia* DNA.

Lane A : EcoRI

Lane B : BamHI

FIG.2.10

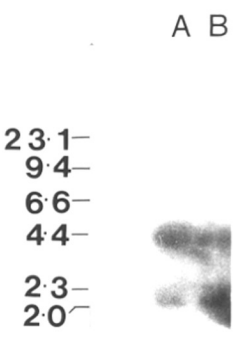


TABLE 2.6

XYLANASE ACTIVITY OF PVX8

	Total Activity (Mili Units)	Total Protein (mg)	Specific Activity (mU/mg)
Fraction I	170	9.765	17.5
Fraction II	100	8.310	18.8

treatment of guanidine hydrochloride (200mM) and Triton X-100 (0.5%), xylanase activity is obtained in the cell free-extracts. There is no induction of xylanase by xylan or IPTG.

2.27.2: Immunodiffusion

Anti-xylanase antibodies were found to cross-react with the cell extract in an Ouchterlony double diffusion. (Fig 2.11).

2.28: RESTRICTION MAPPING OF pVX8

The plasmid was isolated from 100ml of PVX8 culture and was digested with different restriction enzymes. Restriction with EcoRI repeatedly gave a single band of 4.1kb instead of excision of the insert. The digestion of pVX8 with BamHI also resulted into a single band of the same size suggesting the absence of BamHI site in the insert (Fig 2.12a). However, the double digestion of the plasmid with EcoRI and BamHI released the insert (Fig 2.12b). It was therefore concluded that the EcoRI site attached to polylinker is not regenerated. The digestion of pVX8 with HindIII resulted into two fragments of 2.8 and 1.3kbp while that with PstI gave fragments of 2.85 and 1.25kbp respectively (Fig 2.12c). The insert therefore has unique sites for both the enzymes.

PvuII digestion resulted in obtaining four bands of 2.35, 0.95, 0.35 and 0.2kbp respectively (Fig 2.12d).

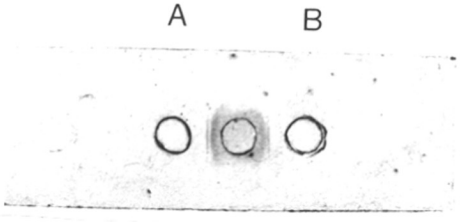
FIGURE 2.11

Immnodiffusion of PVX8 cell extract with anti-xylanase antibodies.

A : PVX cell extract

B : Low molecular weight Chainia xylanase.

FIG. 2.11



pUC8 has two sites of PvuII, one on each side of the polylinker. The distance between first PvuII and BamHI site is 94bp while that of other is 200bp. To determine the exact position of the two PvuII sites in pVX8 insert, PvuII + BamHI double digestion was performed, which gave bands of 2.35kb, 850bp, 350bp, 200bp, and 100bp respectively (Fig 2.12e). It was, therefore, concluded that the first PvuII site in the insert is 850bp and the second site is at a point 1.2 kb apart from the BamHI site in the polylinker. There is no site of BglII and XhoI in the insert. The restriction map of pVX8 is shown in Fig 2.13.

2.29: REORIENTATION OF INSERT

When EcoRI-BamHI fragment of pVX8 insert was eluted from gel and cloned into EcoRI+BamHI digested pUC9, the orientation of the insert with respect to the lacO/P is reversed. The recombinant with reversed orientation was named as PXX8R. EcORI+BamHI digestion of pVX8R plasmid DNA shows two bands corresponding to insert and pUC9 respectively (Fig 2.14). To check the effect of the reorientation on the xylanase activity, PVX8R was grown in 50ml broth and treated as described in section 2.20. PVX8R gives same xylanase activity as that of PVX8 suggesting that the reorientation has no effect on the levels of xylanase gene expression.

FIGURE 2.12

Restriction Digestions of pVX8

FIGURE 2.12a

Lane A : EcoRI

Lane B : Lambda HindIII marker

Lane C : BamHI

FIGURE 2.12b

Lane A : EcoRI+BamHI

Lane B : Lambda HindIII marker

FIG 2·12

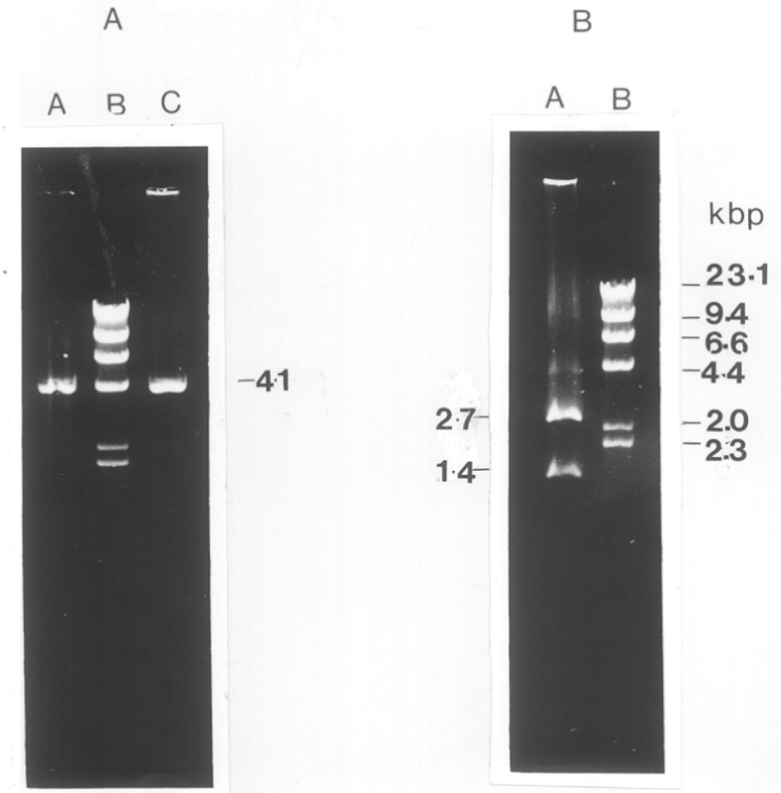


FIGURE 2.12c

Lane A : Lambda HindIII marker

Lane B : EcoRI

Lane C : HindIII

Lane D : PstI

FIGURE 2.12d

Lane A : PvuII

Lane B : Lambda HindIII+EcoRI marker

FIGURE 2.12e

Lane A : PvuII+BamHI

Lane B : Lambda HindIII+EcoRI marker

Lane C : Lambda HindIII marker

FIG. 2·12

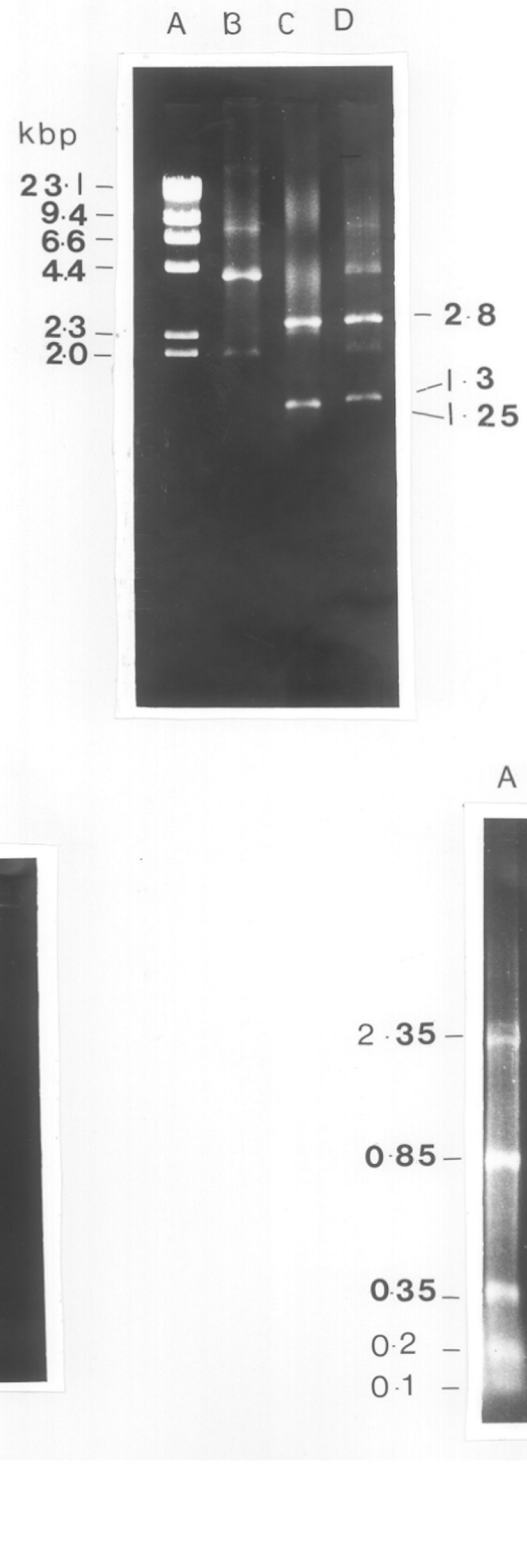

c
A B C D
kbp
23·1
9·4
6·6
4·4
2·3
2·0
2·8
1·3
1·25
d
A B
2·35
0·95
0·35
0·2
e
A B C
2·35
0·85
0·35
0·2
0·1

FIGURE 2.13

Restriction Map of pVX8

EcoRI* represents the EcoRI site that is not regenerated

E – EcoRI
H – Hind III
P – Pst I
B – Bam HI
P_V – Pvu II

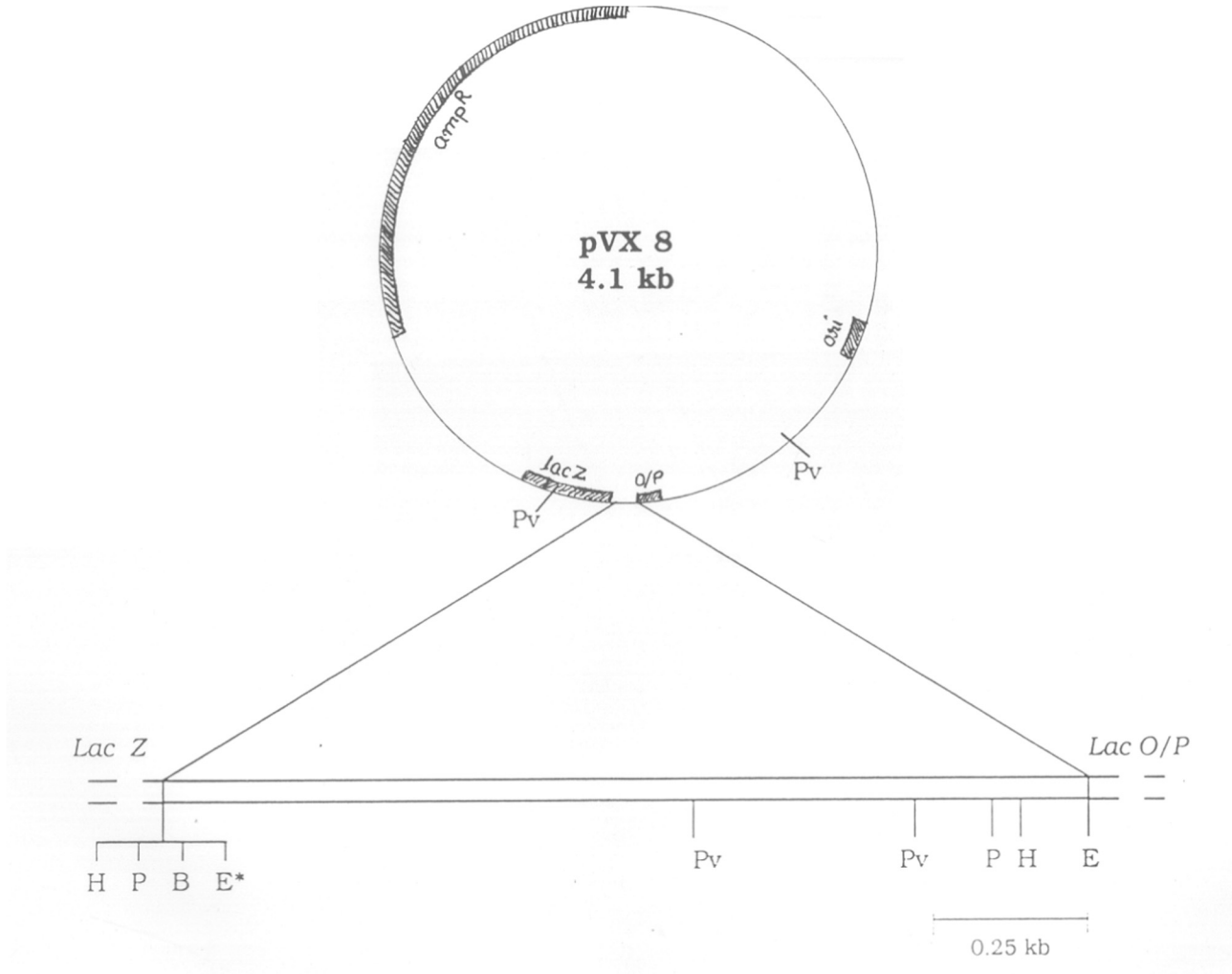

ampR
pVX 8
4.1 kb
ori
lacZ
O/P
Pv
Pv
Lac Z
Lac O/P
H P B E*
Pv
Pv
P H
E
0.25 kb

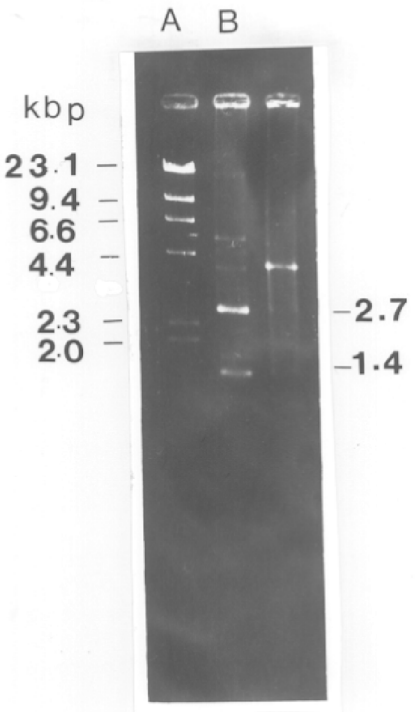
FIGURE 2.14

Restriction of pVX8R

Lane A : Lambda HindIII

Lane B : EcoRI+BamHI

FIG. 2·14



DISCUSSION

2.30: CHOICE OF HOST AND VECTORS

It is of prime importance to choose an appropriate host-vector system for any experiment in molecular cloning . No universally ideal host-vector system exists and a choice depends upon several factors such as the primary objective of cloning, the availability of the probes to screen the desired sequences and the basic requirements for exploiting any organism as a host etc.

In the present work, E. coli was used as a host of choice. Taking into consideration the taxonomical proximity of Chainia with Streptomyces, the latter might have been the ideal host. However, (i) the presence of xylanase gene in S. lividans (6,7), (ii) availability of relatively narrow range of Streptomyces vectors, (iii) possibility of using a wide range of plasmid and bacteriophage vectors with E. coli as a host and (iv) the relatively simple but highly efficient means of introducing chimeric molecules in E. coli have prompted us to use it as a host for cloning.

The diversity of plasmid and phage vectors which are available to use with E. coli as a host, makes it difficult to choose an appropriate vector. In the present work, bacteriophage lambda derived vector, lambda gt10 was used for the construction of genomic library and the plasmid vector pUC8 was used for subcloning. Lambda gt10

is an insertion vector (12) with a maximum insert capacity of 7.2kbp. It has a unique EcoRI site in cI repressor gene of the phage. The digestion with EcoRI, therefore, gives rise to two arms of 32.7kbp and 10.6kbp in length. Cloning in ECoRI site of gt10 leads to an insertional inactivation of cI repressor gene and formation of clear plauqes as compared to turbid plaques of cI^+ parental phages. This is because the parental phages are capable of entering either lytic cycle or dormant lysogeny, while the cI^- recombinants are unable to enter lysogeny due to lack of synthesis of repressor.

In addition, when a mixture of parental phages and recombinants (i.e. both cI^+ and cI^-) is plated on $\underline{hfl}^+$ (High Frequency Lysogeny) strain, cI^+ phages invariably enter into the lysogenic mode, while cI^- recombinants grow well. The selection is based on the fact that hfl strains being protease negative (such as $\underline{himA}^-$), the regulatory proteins cII and cIII are exceptionally stable inside an infected cell and positively regulate the expression of cI repressor from P_{RE} (24,25). Therefore, with the use of both normal (e.g. L87) and $\underline{hfl}^+$ (e.g. NM514) strains, one can calculate the percentage of recombinants in a library. Further amplification of the library on NM514 results into propagation of only recombinants.

However, lambda gt10 is not an expression vector. Therefore, a library constructed in gt10 needs to be

screened with nucleic acid probes. As the complete amino acid sequence of the low molecular weight xylanase from Chainia is available, an oligonucleotide probe corresponding a sequence of to 6 amino acids in the protein was synthesized and used for screening.

2.31: CONSTRUCTION OF Chainia GENOMIC LIBRARY

Considering the genome size of Chainia to be 10^4 kbp, libraries V2 and V3 are complete for all practical purposes. Even in an unamplified library, the background of parental phages is very low. Theoretically, parental gt10 phages should not form plaques on NM514. However, because of spontaneous mutations, some cI^- recombinants might have been generated which are capable of growing on NM514. In the present cloning exercise, this background is very low (i.e. 1 cI^- mutant in approx. 450 cI^+ phages). On the other hand the similar ratios for V2 and V3 are 1.16:1 and 1.09:1 respectively showing that more than 86% and 91% of the phages in unamplified library are recombinants. The presence of insert in the recombinants as evidenced by agarose gel electrophoresis and by plaque hybridization with genomic DNA of Chainia also confirms these results.

2.32: SCREENING OF GENOMIC LIBRARY

There are several methods adopted for screening of xylanase gene from a genomic library. Most of them are

based on the expression of xylanase gene and detection of xylanase activity. However, in the present case, instead of directly screening for the gene product, the library was initially screened with an oligo-probe for the presence of a DNA fragment containing a partial or the complete gene sequence. This strategy was used in view of the following reasons:

1: Lambda gt10 is not an expression vector.

2: The xylanase gene promoter may or may not be recognized in E. coli.

3: As the complete amino acid sequence of the low molecular weight xylanase from Chainia is known, an oligoprobe could be synthesized.

The alternative to this approach could have been to screen gt10 library by heterologous probe or to use gt11 as a vector followed by screening with antibodies. However, generally gt11 library is not amplified thus its long-term storage becomes relatively difficult.

2.33: EXPRESSION OF XYLANASE GENE IN E. coli

The selection of pUC8 as a vector for subcloning is with the anticipation of expression of xylanase gene under the control of β-galactosidase promoter of the vector. pUC8 is a multicopy vector having polylinker between lacO/P and lac-α fragment in addition to the ampicillin resistance marker from pBR322 (13). The selection of recombinants is based on intraallelic (α)

complementation of lacZ M15 mutation in JM105 and lac-α fragment of pUC8 (26). While lac^+ non-recombinants give blue colonies on X-gal plates, lac^- recombinants give white colonies. Generally, it is preferred to clone a desired DNA fragment in a high copy number vector as it facilitates its isolation and purification in large quantities. Due to the high gene dosage effect, a cloned gene product may be overexpressed enabling easy recovery of the recombinant protein.

The copy number of pUC plasmids is significantly higher than that of pBR322 which results into the constitutive lac^+ phenotype on LB medium containing X-gal i.e. appearance of blue colonies of pUC8.JM109 even in the absence of IPTG due to the removal of copy number regulatory regions from the plasmid. In colEI derived plasmids such as pBR322, the copy number is regulated by the hybrid formation of RNAI:RNAII and a small peptide of 66 amino acids named as Rom or Rop which stabilizes this hybrid. In pUC plasmids, rop (rom) gene (27) is absent. In addition, they carry a mutation (G-A transition) one nucleotide upstream of the normal site of initiation of RNAI (28,29). Because of these reasons, copy number of pUC is even higher than that of pBR322. An ultra-high copy number of pUC plasmids may be detrimental to cell metabolism. Therefore, a pBR322 copy number derivative of pUC8 viz. pHG165 is derived in which a copy number control has been recovered (30).

Of the several Streptomyces genes expressed in E. coli, the cloned gene is mostly under the control of vector promoter e.g. neomycin phosphotransferase gene from S. fradiae (31), glnA from S. coelicolor (32), Ptt^R from S. viridochromogenes Tu494 (33), IPNS from S. lipmanii (34). The pac gene of S. alboniger ATCC 12461, when subcloned in pUC19, PAC (puromycin N-acetyl transferase) activity was detected only in one of the two possible orientations and is stimulated by IPTG (35). Similarly, CAT gene of S. acrimycini is shown to be expressed only under the control of tet promoter of pBR322 (36).

On the other hand, in case of endoH from S. plicalus, it is predicted that it can be expressed even from its own promoter in addition to the vector promoter (37).

The xylanases from diverse sources have been cloned in E.coli (Table 2.7) and in most of the cases, the cloned gene exhibits reduced levels of expression. However, in Clostridium acetobutylicum (38), Bacteroides ruminicola (39), alkalophilic aeromonas (40) and B. circulans (41,42), hyperexpression is detected. In case of B. circulans a fragment of 0.3kbp is detected which behaves like eukaryotic enhancer elements as the expression levels are influenced by the location of fragment in positions both upstream and downstream of the

TABLE 2.7

EXPRESSION OF HETEROLOGOUS XYLANASE GENES IN *E. coli*

Source	Expression of cloned gene/s	Ref
B. polymyxa	Expressed in absence of xylan	52
Bacillus pumilus	5 to 50 times hyperexpression in one of the orientations.	44,54
B. circulans	Hyperexpression due to enhancer like sequence	41,42
Ruminicoccus flvefaciens	*xynA* expression is reduced in presence of glucose	46
Clostridium Thermocellum	Truncated protein shows activity	48,57
Clostridium acetobutalicum	increase in specific activity	38
Bacteroides ruminicola 23	a] 1.2 times Higher. b] From endogenous promoter.	39

TABLE 2.7 (Contd)

Source	Expression of cloned gene/s	Ref
Bacteroides ruminicola D31d	under control of endogenous promoter	43
Bacteriodes succinogenes	10 times higher under endogenous promoter	56
Alkalophilic Aeromonas 212	80 times higher.	40
Thermomonospora fusca	Low levels in the absence of vector promoter	50
Cellulomonas sp.	a] No activity in the absence of xylan b] No induction by IPTG	45
Pseudomonas flurescens subsp. cellulosa	No effect by xylan and glucose	53
Caldocellum saccharolyticum	55 times higher Under control of vector promoter.	55

xylanase gene (42). Deletion of this fragment results in the drastic drop in intracellular activity. Similarly, E. coli harboring xylanase gene from Bacteroides ruminicola 23 produces higher activity than the donor strain in the absence of IPTG (39). In the other strain of Bacteroides ruminicola (D31d), the expression is observed in both the orientations (43).

Panbangred et al have observed that xylanase activity is increased by 5 to 50 times after reorientation in pBR322 suggesting that the hyperexpression is due to the control of expression by tet promoter of the vector (44).

The failure of induction of the cloned Chainia xylanase gene by IPTG may be attributed to two main reasons. Firstly, the possibility of titering out of lac repressor by multicopy vector cannot be ruled out. However in such a case, no expression is possible in the other orientation. Secondly, expression may not be under the control of β-galactosidase promoter. As the level of expression of the cloned gene remains unchanged in both the orientations, the second possibility seems to be more likely and suggests that the observed expression is because of recognition of xylanase promoter in E. coli. These results are consistent with our studies on the isolation of Chainia promoters that are functional in E. coli.

In case of Cellulomonas and Ruminicoccus

flavefacienss, xylan induction and glucose repression of cloned xylanase gene is reported (45, 46). However, xylan has no effect on xylA of Chainia suggesting the absence of functional regulatory gene in the PVX8 in view of the fact that xylanase expression is xylan inducible in Chainia.

Though the weak expression of xylA promoter in E. coli is likely to be the main reason for the low levels of xylanase activity, the other reasons such as the distance between lacP and xylA, or bias for codon usage may also have an adverse effect on the expression levels. In case of Streptomyces, high bias against TTA codon for leucine is reported (47). Out of 19,460 codons in 63 Streptomyces genes screened, TTA codon appears only four times. On the other hand, the other codons for leucine are abundant. However, no information is available on codon usage in Chainia.

2.34: FUTURE SCOPE OF THE WORK

Chainia secretes two distinct xylanases. The xylA was screened by its homology with the oligoprobe corresponding to the low mol wt xylanase gene. It is not clear whether these two xylanases are expressed by two distinct genes as in case of S. lividans (8). However, in case of cloned xynZ gene of Clostridium thermocellum, two xylanases are presumed to be proteolytic products of the

larger polypeptide. In addition, in spite of the lack of more than half of the sequence encoded by xynZ, the truncated protein is highly active on xylan (48). On the other hand three different xylanase genes have been reported in the same species by the other researchers (49). As the size of low molecular weight xylanase from Chainia is quite small, it is unlikely that it will give rise to further active fragments. However, presence of common transcript for both the xylanases cannot be ruled out. Screening for the other DNA fragments representing the xylanase gene/s and studying their homology by hybridizations and sequencing can throw some light on these aspects.

For biotechnological exploitation of xylanase, hyperexpression of the cloned gene will be desirable. As described previously, transfer of the same fragment into Streptomyces lividans and/or Chainia may result in the hyperexpression. When xylanase gene from thermomonospora fusca was transferred to S. lividans, 20-fold higher activity than produced by E. coli clones was observed (50). We have shown the presence of heat attenuable restriction system in Chainia by the transformation of Chainia protoplasts with Streptomyces lividans broad host range vector pIJ702 (51). However, two main problems which may arise in this respect are i] presence of high xylanase activity in S. lividans and ii] poor knowledge

regarding the recombination system in Chainia. There are chances that the cloned fragment will be integrated into Chainia chromosome by homologous recombination which will neutralize gene dosage effect. Better understanding of the molecular regulation in Chainia will be helpful for this purpose.

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CHAPTER III

GENETIC TRANSFORMATION OF *CHAINIA*:

PRESENCE OF HEAT ATTENUABLE RESTRICTION SYSTEM

ABSTRACT

To investigate restriction-modification system in Chainia, the protoplasts of Chainia were transformed by Streptomyces broad host range vector pIJ702. The transformation efficiency was very poor, suggesting the presence of operative restriction system in Chainia. When the plasmid DNA isolated from Chainia was used to transform Chainia protoplasts, increase of transformation efficiency by hundred fold was observed suggesting that the plasmid DNA is suitably modified. Similarly, when Chainia protoplasts were heated at 42°C for 10 min just prior to transformation, even the plasmid isolated form S. lividans yielded high transformation efficiency. The results show the presence of heat attenuable restriction system in Chainia.

INTRODUCTION

The molecular cloning and expression of Chainia xylanase gene in E. coli has been described in chapter II. It reveals that the cloned gene is expressed in E. coli and probably its expression is through its own promoter. As discussed earlier, there are several approaches through which the levels of expression can be enhanced. One of these approaches is self-cloning in Chainia so that cumulative effect of high copy number, recognition of regulatory elements, post-translational modifications (if any) and excretion will result into hyperproduction of enzyme. Though this approach appears very attractive, several problems have to be overcome before using any new genus as a host. The availability of appropriate vectors, ways of introducing vector and chimeric molecules inside a host cell and the presence of restriction barrier in a host are some of these problems. Among the prokaryotes, Actinomycetes have been widely exploited for their industrial potential. With the advent of polyethylene glycol (PEG)-mediated transformation of Streptomyces protoplasts with plasmids, numerous genes have been successfully cloned in S. lividans (1). Though Escherichia coli is the most commonly used cloning host, heterologous proteins are generally retained within the cells as insoluble inclusion bodies. Therefore, Streptomyces is receiving increasing attention as a

potential protein-secreting host for molecular cloning (2). Recently, intergeneric transformation with Streptomyces plasmid vectors of Micromonospora (3), Saccharopolyspora erythraeas (4), Thermomonospora fusca (5), Amycolatopsis orientalis (6), and Arthrobacter (7) have been reported.

As Chainia is closely related to Streptomyces (8,9,10) it was proposed to study protoplast formation, PEG- mediated transformation, restriction-modification, and the maintenance of the Streptomyces broad-host-range vector pIJ702 in Chainia. The restriction modification system of Chainia was examined by using native and modified plasmid DNA to transform normal and heat-treated protoplasts.

MATERIALS AND METHODS

3.1: CHEMICALS, MATERIALS AND STRAINS

Polyethylene glycol (molecular weight 1000) (PEG-1000) and lysozyme was obtained from Sigma, U.S.A. All the chemicals were of analytical grade. Thiostrepton was a kind gift of E.R. Squibb and Sons Princeton N. J., U.S.A.

Streptomyces lividans 3131 harboring pIJ702 (11) and S. lividans TK64 were kindly supplied by Dr. N. K. Notani, B.A.R.C. Bombay.

3.2: MEDIA AND BUFFERS

YEME BROTH

Yeast extract	0.3%
Malt extract	0.3%
Peptone	0.5%
Dextrose	1.0%

NCCP MEDIUM

Dextrose	1.0%
NaCl	0.5%
Peptone	0.5%
Yeast Extract	0.5%
$CaCl_2$	0.2mg%

pH 7.2

TRACE ELEMENT SOLUTION (per litre)

ZnCl2	40mg	$Na_2B_4O_7.10H_2O$	10mg
$FeCl_3.6H_2O$	200mg	$(NH_4)_6Mo_7O_{24}.4H_2O$	10mg
$CuCl_2.2H_2O$	10mg	$MnCl_2.4H_2O$	10mg

R2YE Medium (Per Litre)

Sucrose	103.0 g
K_2SO_4	0.25g

$MgCl_2.6H_2O$	10.12g
Glucose	10.0g
Casaminoacids	0.1g
Trace element solution	2.0ml
yeast extract	5.0g
Tris buffer	5.73g
Bacto Agar	22g

After autoclaving following pre-sterlilized solutions were added to 100 ml medium:

KH_2PO_4 (0.5%)	1ml
$CaCl_2.2H_2O$ (5M)	0.4ml
L-proline (20%)	1.5ml
NaOH (1N)	0.7ml

P (PROTOPLAST) BUFFER

Sucrose	103g
K_2SO_4	0.25g
$MgCl2.6H_2O$	2.02g
trace element solution	2ml

Volume adjusted to 800ml

After autoclaving following solutions were added per 80ml of buffer

KH_2PO_4 (0.5%)	1ml
$CaCl2.2H_2O$ (3.68%)	10ml
Tris Buffer (5.73%; adjusted to pH7.2)	10ml

3.3: ISOLATION OF PLASMID DNA

Streptomyces lividans (pIJ702) and Chainia (pIJ702) were grown for 48 h in 500 ml baffled flasks containing 100 ml of NCCP and YEME medium, respectively, with 25 μg/ml thiostrepton. The plasmid was isolated by an alkaline lysis procedure as follows (12).

Approximately 1g (wet weight) of cells were suspended in 5ml lysozyme solution (2mg/ml in GET) and incubated at 37°C for 30min. The suspension was gently mixed three-four times during incubation. 2.5ml NaOH/SDS (0.3M and 2% respectively) was added and mixed thoroughly followed by incubation at 55°C for 35min. 800μl acid phenol/chloroform was added, mixed thoroughly by vortexing and centrifuged for 10min at 5000rpm. The clear supernatant liquid was separated and DNA was precipitated by addition of 0.1 volume of sodium acetate (700μl) and equal volume (7ml) of isopropanol. After centrifugation, DNA was dissolved in 500μl TE and treated with RNase followed by treatment with equilibrated phenol/chloroform, two washes of chloroform and DNA was reprecipitated as above.

3.4: PREPARATION OF PROTOPLASTS

The protoplastation of Chainia and Streptomyces lividans was carried out according to Hopwood et al (13). Chainia sclerotia or S. lividans TK64 spores were inoculated into 25ml of YEME medium containing 34% (w/v)

sucrose, 5mM $MgCl_2$ and 0.5% glycine in 250 ml baffled flasks. After incubation of 36 h at 30°C on a rotary shaker, the cultures were centrifuged and the mycelium was washed twice with 10.3% sucrose. The washed mycelium was resuspended in P buffer (4 ml) containing lysozyme (1mg/ml). The suspension was triturated 3-4 times after an incubation of 30min at 28° C, and then allowed to stand for 15min, before 9 ml of P buffer was added. The suspension was filtered through cotton to remove mycelial fragments.

The protoplasts recovered by centrifugation (3000 rpm for 7 min) were washed twice in 5 ml of P buffer and resuspended in 1ml of the same buffer. They were then diluted with P buffer and plated on R2YE agar plates (at the concentration of $5x10^3$ protoplasts/plate). The proportion of un-protoplasted colony forming units was determined by treatment with 0.01% SDS before plating.

3.5: TRANSFORMATION OF PROTOPLASTS

Approximately 10^8 protoplasts (as per haemocytometer count) were used for each transformation experiment. The plasmid DNA (100ng) up to 20μl was added to the protoplast suspension followed by immediate addition of 0.5ml of 25% PEG-1000 in P buffer. It was mixed by trituration and 5ml of P buffer was added. The suspension was centrifuged at 3000rpm for 10min and the

pellet was resuspended in 1ml P buffer. 0.1ml aliquot of this transformation mixture was plated on each R2YE plates and incubated at 30°C. After 18h incubation, the plates were overlaid with 3ml NCCP soft agar containing 25μg/ml thiostrepton.

3.6: HEAT TREATMENT OF PROTOPLASTS

Approximately 10^8 protoplasts were incubated in water bath at 28°C, 42°C, 45°C and 48°C respectively for 10 min. After heat treatment, they were immediately subjected to transformation as above. An aliquot of protoplasts was removed before transformation for studying regeneration efficiency.

3.7: REGENERATION OF PROTOPLASTS

Table 3.1 shows the haemocytometer counts and viable counts of Chainia and S. lividans protoplasts. Chainia protoplasts regenerated at about 5% efficiency while for S. lividans protoplasts the efficiency was 10%. The frequency of un-protoplasted colony-forming units was lower than 1% as determined by treatment with 0.01% SDS prior to plating on R2YE plates.

3.8: TRANSFORMATION

Streptomyces broad host range plasmid vector pIJ702 was used to transform both Chainia and S. lividans protoplasts. Figure 3.1 shows the transformation efficiency of both the strains. As evident from the Figure, Chainia protoplasts were transformed with very poor efficiency (5-10 transformants per microgram of DNA) as compared to that of S. lividans ($>10^4$ transformants/μg DNA). However, the transformation efficiency of Chainia protoplasts is increased by more than 100 folds (1×10^3 /μg DNA) when pIJ702 was isolated from Chainia transformants and used for subsequent transformation. The S. lividans protoplasts can be transformed with the equal efficiency by pIJ702 (Chainia).

TABLE 3.1

PROTOPLASTATION AND REGENERATION OF *CHAINIA* AND *STREPTOMYCES LIVIDANS* TK64

Organism	Haemocytometer Count (Cells/ml)	Viable Count (CFU*/ml)	Regeneration Efficiency (%)
S. lividans	2×10^9	1.68×10^8	10
Chainia	2.02×10^9	9×10^7	5

FIGURE 3.1

Transformation efficiency of *Chainia* and *S. lividans* TK64 protoplasts by pIJ702

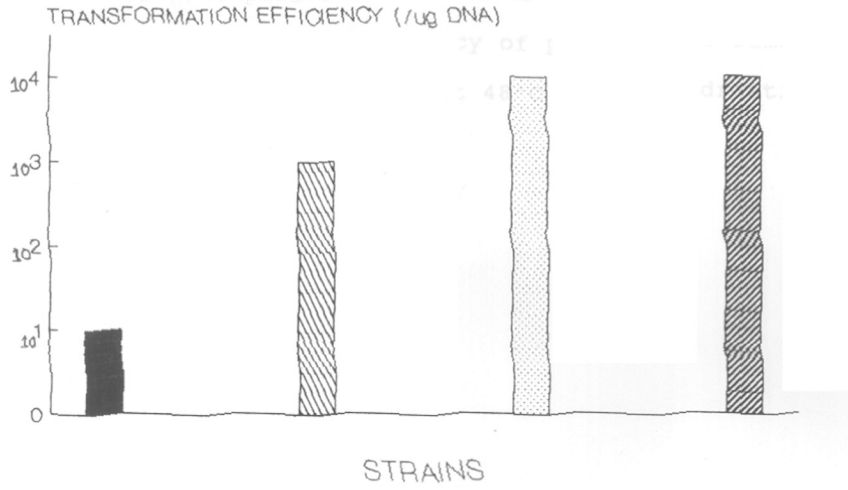
A] *Chainia* protoplasts transformed by pIJ702 isolated from *S. lividans*.

B] *Chainia* protoplasts transformed by pIJ702 isolated from *Chainia*(pIJ702)

C] *S. lvidans* protoplasts transformed by pIJ702 isolated from *S. lividans*.

D] *S. lvidans* protoplasts transformed by pIJ702 isolated from *Chainia*(pIJ702)

FIGURE 3.1



3.9: EFFECT OF HEAT TREATMENT

When Chainia protoplasts were subjected to heat treatment and used for transformation with pIJ702 isolated from S. lividans TK3131, there is a drastic increase in the transformation efficiency (Fig 3.2). There is no difference in the transformation efficiency of protoplasts heated at 42°C and 45°C respectively. The regeneration efficiency of protoplasts remains constant up to 45°C. However at 48°C it drops drastically (<1%) (Fig 3.3).

FIGURE 3.2

Efficiency of transformation of heat treated Chainia protoplasts with pIJ702 isolated from TK3131.

FIGURE 3.2

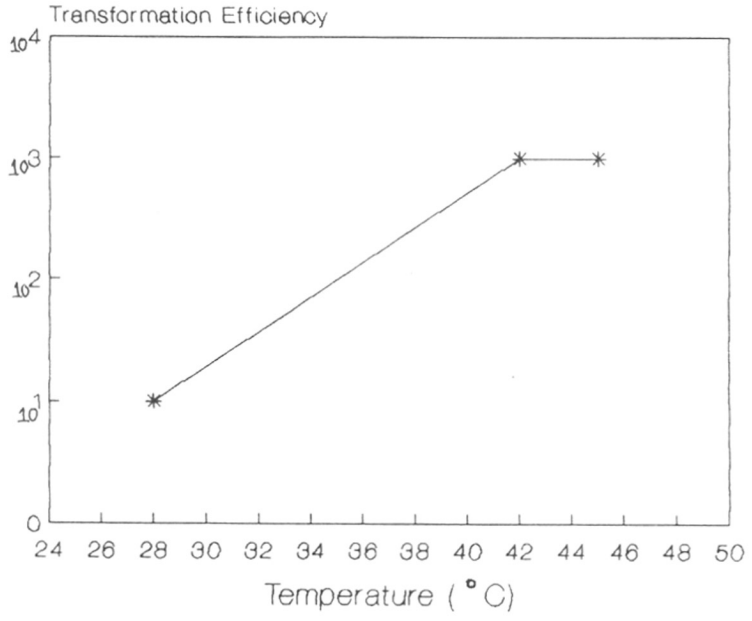
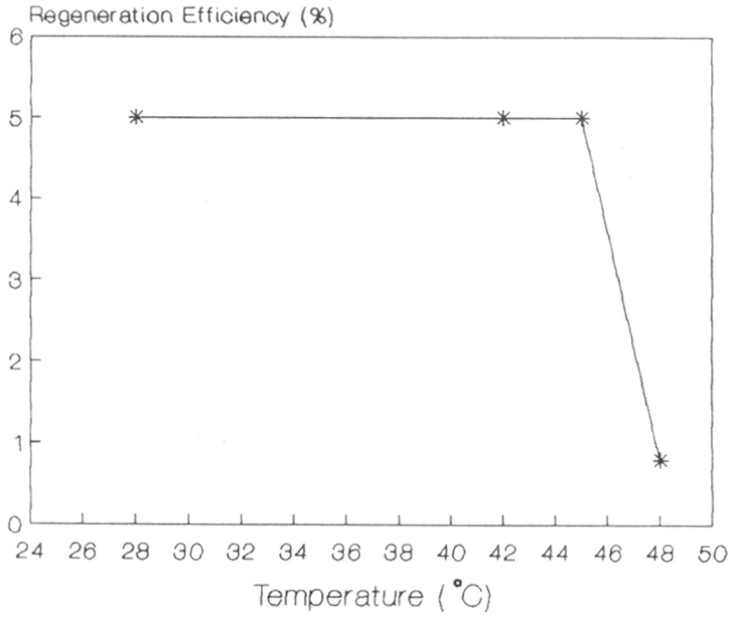

Transformation Efficiency
10^4
10^3
10^2
10^1
0
24 26 28 30 32 34 36 38 40 42 44 46 48 50
Temperature (°C)

FIGURE 3.3

Regeneration efficiency of heat treated

Chainia protoplasts

FIGURE 3.3


Regeneration Efficiency (%)
6
5
4
3
2
1
0
24 26 28 30 32 34 36 38 40 42 44 46 48 50
Temperature (°C)

DISCUSSION

3.10: PROTOPLASTATION AND REGENERATION

The standard protocols available for the preparation of S. lividans protoplasts were used during this study. Though the regeneration efficiency of Chainia protoplasts is comparatively low, these studies have shown that the techniques (such as protoplast formation and their regeneration) used for S. lividans can be applied to Chainia. In both S. lividans and Chainia, un-protoplsted colony forming units were less than 1% as detected by 0.01% SDS treatment. This concentration of SDS results in the lysis of protoplasts but has no effect on mycelium.

3.11: TRANSFORMATION

The transformation efficiency of Chainia protoplasts using pIJ702 isolated from S. lividans, is very low. Though several genetic and technical reasons can be attributed to this observation, one of the most important reason may be the presence of restriction system in Chainia. The transformation efficiency is increased by more than hundred folds when a plasmid isolated from transformed Chainia was used. It confirms the presence of restriction-modification system in Chainia. While in the first set of experiments (where pIJ702 isolated from S. lividans was used), the plasmid DNA cannot be established in Chainia because of its

operative restriction system. Only those molecules which are modified before exposure to restriction, can confer thiostrepton resistance to Chainia. However, once modified, it can be transformed with higher efficiency as the restriction barrier is overcome. The presence of a restriction-modification system in Chainia is a phenomenon commonly observed in Streptomyces (14). When pIJ702 isolated from S. lividans was used to transform Amycolaptosis orientalis protoplasts 5.0 X 10^4 transformants per microgram of DNA was obtained. On the other hand, the efficiency increased to 10^6, when the plasmid isolated from A. orientalis was used. Therefore, it appears that the relatively small pIJ702 DNA is moderately restricted in A. orientalis (6, 15). However, in case of Chainia the original transformation efficiency is quite low suggesting the presence of a strong restriction system.

3.12: HEAT ATTENUATION OF RESTRICTION

When Chainia protoplasts were subjected to heat treatment for 10 min at 42°C and transformed with pIJ702 isolated from S. lividans TK3131, the transformation efficiency was equal to that observed with pIJ702 isolated from Chainia transformants (Figure 3.2). It shows that the heat treated Chainia protoplasts can be transformed efficiently even by an unmodified plasmid DNA. This evidence that the restriction barrier in

Chainia is inactivated by subjecting the protoplasts to elevated temperature is similar to the heat-mediated restriction attenuation observed in Streptomyces clavuligerus and Streptomyces tendae (16,17).

There is no difference in the regeneration efficiency and transformation efficiency of the Chainia protoplasts heated at 42°C and 45°C (Fig 3.3). In case of S. tendae and S. clavuligerus, the optimum temperature for heat attenuation was 50°C for 30 minutes and 45°C for 10min respectively. However, in case of Chainia, there is drastic reduction in the regeneration efficiency when the protoplasts were heated at 48°C. This may primarily be due to the inability of the protoplasts to withstand this temperature.

3.13: CHAINIA AS AN ALTERNATIVE HOST

Among the genus Streptomyces, although S.lividans is widely exploited as a cloning host, a few reports are available regarding the use of other streptomycetes as a host (18,19). Chainia (NCL 82-5-1) is known to produce several industrially important enzymes (20,21). It will be, therefore, desirable to develop this strain as a cloning host. However, There are several unanswered questions regarding Chainia which need to be before developing it as a host. The Hosts used for molecular cloning are, as a rule, restriction deficient (r^-) mutants. This is of prime importance for the smooth

passage and propagation of an exogenous DNA fragment. With the occurrence of restriction system in Chainia, It is important to isolate similar mutants. Restriction modification system, several of them associated with specific type II restriction endonucleases, are widespread in the genus Streptomyces (21). Matsushima et al have isolated S. fradiae mutants defective in restriction by sequential selection for increased transformability by several plasmid DNAs. From the number of mutations and selective cycles required to eliminate most restriction systems, it was estimated that wild type S. fradiae expressed at least five restriction system (22). Though self-cloning experiments can be performed even on the r^+ hosts using modified plasmid, the problem of restriction will persist while transferring Chainia genes previously cloned in other hosts.

Streptomyces broad host range vector, pIJ702, is used in the present study. The results reported here establish that the transcription and translation systems in Chainia recognize the Streptomyces regulatory signals associated with the expression of thiostrepton resistance gene of pIJ702. However, pIJ702 may not be an ideal vector for all types of cloning experiments. While S. gramaticolor protoplasts were found to be trnasformable by pIJ350, no transformants were obtained when pIJ2 was used (23). Successful introduction and stable maintenance of other Streptomyces vectors will be useful in this

respect.

Very little is known about the recombination system in Chainia which may result in deletions in inserts, nullifying gene dosage effect and inability to excise insert (due to integration of chimeric molecule into the chromosome) etc. Detailed study on these aspects will be useful for the future developments.

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Chapter IV

Molecular Cloning And Expression Of *Chainia* Promoter Sequences In *Escherichia Coli*

ABSTRACT

To ascertain whether Chainia genome has any sequences which initiate transcription in E. coli, Chainia DNA, partially digested with sau 3AI and size fractionated (50-500bp), was ligated with BamHI restricted and dephosphorylated pJAC4 and used to transform into E. coli DH1. The plasmid pJAC4 has a Km^R gene and promoterless β-lactamase gene as a reporter gene of promoter activity. The Km^R transformatnts were replica plated on different concentrations of ampicillin (0-30μg/ml)>. The origin of insert in the amp^R was confirmed by colony hybridization with labelled genomic DNA of Chainia. The expression of promoters in four clones (PCP3, PCP4, PCP6 and PCP9) was analyzed by determining β-lactamase activity and LD_{50} values. The results show that out of four promoters, PCP6 is the strongest with LD_{50} value of 16μg/ml of ampicillin, while PCP9 is the weakest (LD_{50} value 7μg/ml). There is a direct correlation between the LD_{50} values and β-lactamase activity of these recombinants.

INTRODUCTION

Irrespective of the controversy regarding the taxonomic status of Chainia, its close proximity with Streptomyces genus suggests that the molecular organization of Chainia may be more or less similar to Streptomyces. As organisms of both the genera undergo complex cycle of morphological differentiation like formation of aerial hyphae and sporulation (in Streptomyces) and sclerotial formation (in Chainia), the strategies for gene regulation in both the organisms are likely to be diverse and perhaps novel.

Transcription initiation is a level of control at which a variety of regulatory mechanisms might exist. The identification and characterization of components and signals involved in this process have been the subject of investigation in variety of bacterial species. These studies have revealed the presence of two conserved sequences termed as -10 and -35 region (1). These sequences represent a major class of prokaryotic promoters. Although typical prokaryotic promoter sequences seem to be present in each of the bacterial genera studied, many other distinctly different promoter sequences have been recognized in genes expressed during heat shock response in E. coli (2) or sporulation in B. subtilis (3,4).

The divergence in promoters is generally

accompanied with the heterogeneity in RNA polymerase holoenzymes. The holoenzymes containing alternative sigma factors have different specificities and thus can be used to transcribe from different sets of promoters. Consequently, a cell with an ability to synthesize different sigma factors has a potential mechanism to control the expression of diverse sets of genes.

The tremendous diversity of Streptomyces promoters and heterogeneity in RNA polymerase can be correlated with each other. It may permit co-ordinate regulation of sets of genes and operons that are transcribed from cognate promoters in response to particular environmental conditions (5). Transcription of dagA by at least three different forms of RNA polymerase holoenzymes represents a unique case in this respect (6). During the exponential growth, the transcription of dagA occurs mainly from dagAP1 and dagAP4 and is subject to induction by agar and repression by glucose. In the stationary phase, high level of expression from dagAP2 and dagAP3 are observed (7)

The advances in molecular cloning techniques facilitate the analysis of gene regulation. The various signals and components involved in the process can be cloned and studied in detail. With the use of promoter probe vectors, in vivo promoter activity can be assessed. In addition, the development of fine analytical techniques such as promoter probing, S1 mapping, primer

extension. run-off transcription, DNaseI protection and *in vitro* mutagenesis have led to pin point the promoter sequences involved in the transcription initiation and their interaction with RNA polymerase\s.

Typical *E. coli* promoter is recognized by σ^{70} associated RNA polymerase holoenzyme. However, even in *E. coli*, some other minor forms of RNA polymerase holoenzyme do exist which regulate transcription of some specific genes like that of heat shock proteins (2).

Heterologous gene expression in *E. coli* thus primarily depends on the recognition of promoters by σ^{70} associated holoenzyme. As *Streptomyces* DNA is highly G+C rich, initially there were a lot of speculations regarding the expression of *Streptomyces* genes in *E. coli*. Horinouchi *et al* have reported the failure of expression of *Streptomyces* genes in *E. coli* (8). However, later on, by shot-gun cloning, Jaurin and Cohen have has shown that *Streptomyces* does contain *E. coli* type A+T rich promoters capable of being recognized by *E. coli* RNA polymerase (9,10). As increasing number of *Streptomyces* promoters are being analyzed, it is now well established that *Streptomyces* possess some, but not all, *E. coli* type promoters. It has been estimated that there are more than 200 *E. coli* type promoters in *S. lividans* chromosome (9).

Though a lot of information is now available regarding the *Streptomyces* promoters, there is no

knowledge regarding the nature of Chainia promoters. In this respect, it is proposed to isolate a few Chainia promoter sequences functional in E. coli using gene fusions. The approach used was a shot-gun cloning of small DNA fragments in E. coli using the promoter-probe vector pJAC4. The details regarding molecular cloning and expression of Chainia promoters in E. coli are described in this chapter.

MATERIALS AND METHODS

4.1: CHEMICALS, MATERIALS AND ENZYMES

Plasmid pJAC4 and chromogenic substrate for β-lactamase activity viz. nitrocefin were kindly provided by Dr. B. Jaurin and Dr. M. Forsman, Sweden. All other chemicals, enzymes and kits were as described earlier (section 2.1).

4.2: ISOLATION OF CHAINIA PROMOTERS

4.2.1: Digestion of Chainia DNA with Sau3AI and size fractionation

High molecular weight Chainia DNA was subjected to digestion with different concentrations of Sau3AI so as to determine the optimum ratio of DNA to enzyme. After standardization of restriction conditions 10μg DNA was digested with Sau3AI (0.5U/μg DNA) for 15min. The reaction was terminated by addition of EDTA to a final concentration 10mM). The digested DNA was loaded on LMT agarose gel (1.2%). After completion of electrophoresis, the gel was cut so as to elute DNA in the size range of 50 to 500bp and purified on Elutip-D column as described in section 2.9.2.

4.2.2: Screening for recombinants showing promoter activity

The size fractionated Chainia genomic DNA was ligated with BamHI cut and dephosphorylated pJAC4 and E. coli DH1 competent cells were transformed with the ligation mixture. The transformants were initially plated

on LB plates containing kanamycin (50μg/ml) and subsequently replica plated on kanamycin plus different concentrations of ampicillin (0 to 25μg/ml). The colonies grown on the different concentrations were picked up and stored on LB plates containing kanamycin plus 5μg/ml ampicillin. The origin of inserts in the recombinants was confirmed by colony hybridization.

4.3 EXPRESSION OF PROMOTERS IN *E. coli*.

4.3.1: Preparation of cell extracts

The recombinants were grown overnight at 37°C in 5ml LB containing kanamycin (50μg/ml). The cells were separated from the growth medium by centrifugation at 12,000g for 15min at 4°C. The pellet was resuspended in 1ml lysozyme solution (2mg/ml) and incubated at 37°C for 30min. On centrifugation, the supernatant was estimated for β-lactamase activity.

4.3.2: Determination of enzyme activity

β-lactamase activity was determined by using nitrocefin as a substrate (11). The method is based on the spectrophotometric measurement of the rate of change of absorption associated with β-lactam ring of nitrocefin as a result of enzymatic reaction which leads to decrease in absorption at 386nm with simultaneous increase at 482nm. The reaction mixture contained nitrocefin (51.6μg/ml), suitably diluted enzyme and 50mM phosphate buffer pH 7, in a total volume of 1ml. The change in

absorption was recorded spectrophotometrically.

The activity of the enzyme is expressed as micromoles of the substrate destroyed per minute per milliliter of enzyme. The calculation of the activity is based on the fact that 10^{-4} M nitrocefin produced a decrease in O.D. of 1.03 at 386nm after complete destruction.

4.3.3: Measurement of ampicillin resistance levels

The level of ampicillin resistance conferred by recombinant plasmids in E. coli was measured by plating out approximately 200 to 600 cells in the mid-log phase on LB agar containing kanamycin (50μg/ml) and different concentrations of ampicillin (0-30μg/ml). The number of surviving colonies was counted after an overnight incubation at 37°C and the concentration of ampicillin required to kill 50% of the colonies as determined graphically was termed as an LD_{50} value.

4.4: CLONING OF *CHAINIA* DNA IN pJAC4

pJAC4 has a kanamycin resistance gene, origin of replication of pBR322 and a promoterless β-lactamase gene (ampC) of *E. coli* as a reporter gene (Fig 4.1) (12). It has a polylinker containing unique restriction sites for BamHI, CIaI, EcoRI, SacI, and XbaI. β-lactamase gene is expressed when a DNA fragment with a promoter activity is inserted in the correct orientation into one of the cloning sites. Cloning of *Chainia* DNA in pJAC4 gave rise to approximately 5000 kanamycin resistant transformants which when replica plated on LB plates containing 10μg/ml ampicillin yielded 12-13 colonies. The hybridization of *Chainia* DNA with the ampicillin resistant clones confirmed the origin of inserts (Fig 4.2).

4.5: EXPRESSION OF CHAINIA PROMOTERS IN *E. coli*

The four promoters (pCP3, pCP4, pCP6 & pCP9) showed significantly higher LD_{50} values than *E. coli DH1*(pJAC4). However, they differed in their relative strengths as evident from the LD_{50} values. (Fig.4.3, Table 4.1). pCP9 is the weakest among the four promoters. Expression of the cloned promoters was also monitored by quantitative estimation of β-lactamase activity elicited by *E. coli* (fig 4.4). It is noteworthy that as anticipated, there is a direct correlation between the LD_{50} values and the enzyme activity (Table 4.1).

FIGURE 4.1

The Map of pJAC4

FIG. 4.1

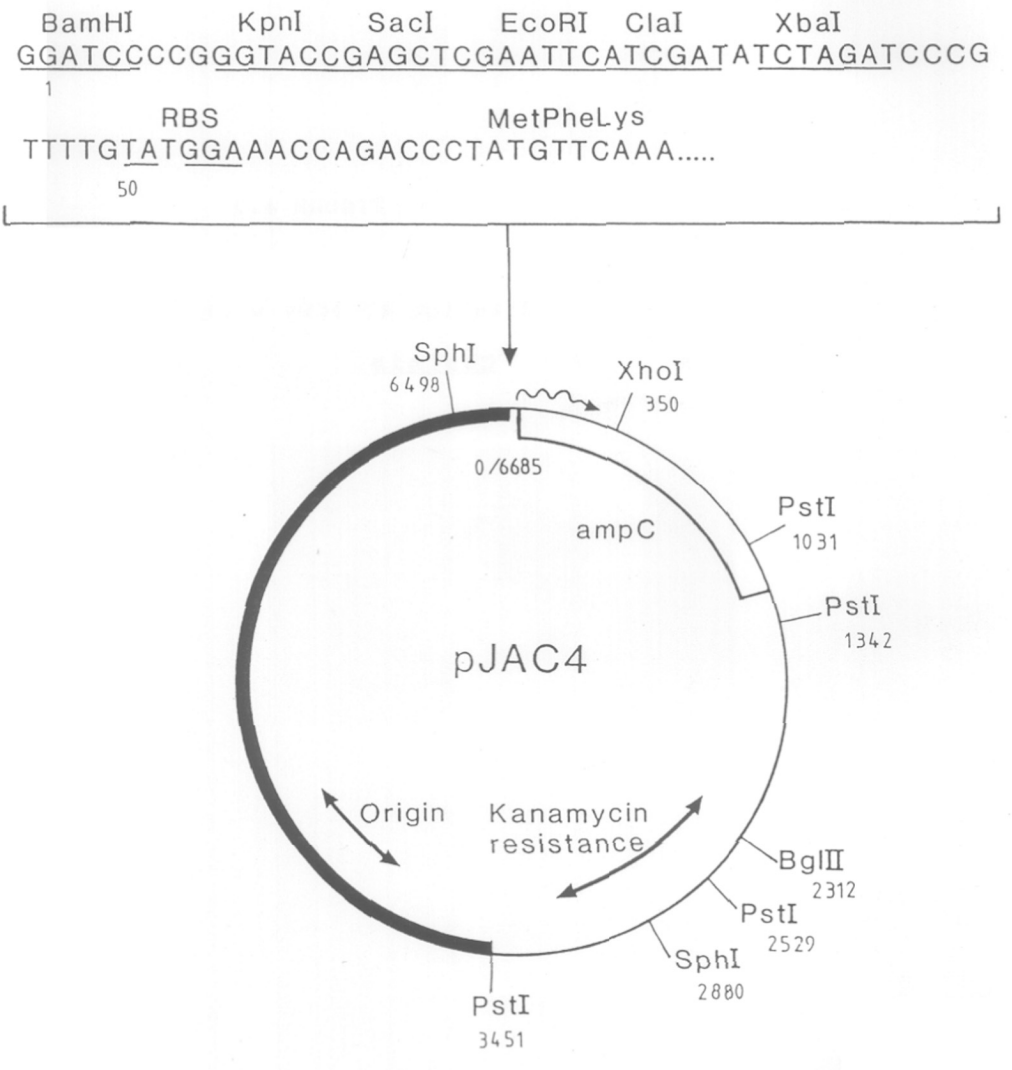
BamHI
KpnI
SacI
EcoRI
ClaI
XbaI
GGATCCCCGGGTACCGAGCTCGAATTCATCGATATCTAGATCCCG
1
RBS
MetPheLys
TTTTGTATGGAAACCAGACCCTATGTTCAAA.....
50
SphI
6498
XhoI
350
0/6685
PstI
1031
ampC
PstI
1342
pJAC4
Origin
Kanamycin
resistance
BglII
2312
PstI
2529
SphI
2880
PstI
3451

FIGURE 4.2

Colony hybridization of PCPs with ^{32}P labelled genomic DNA of *Chainia*.

FIG 4·2

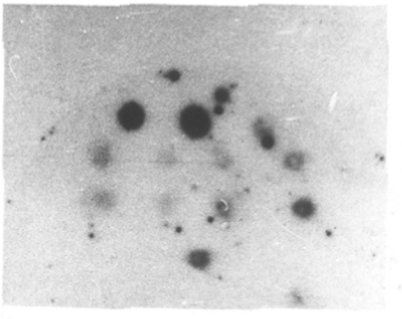

FIGURE 4.3

Ampicillin resistance levels of PCPs.

FIG. 4.3

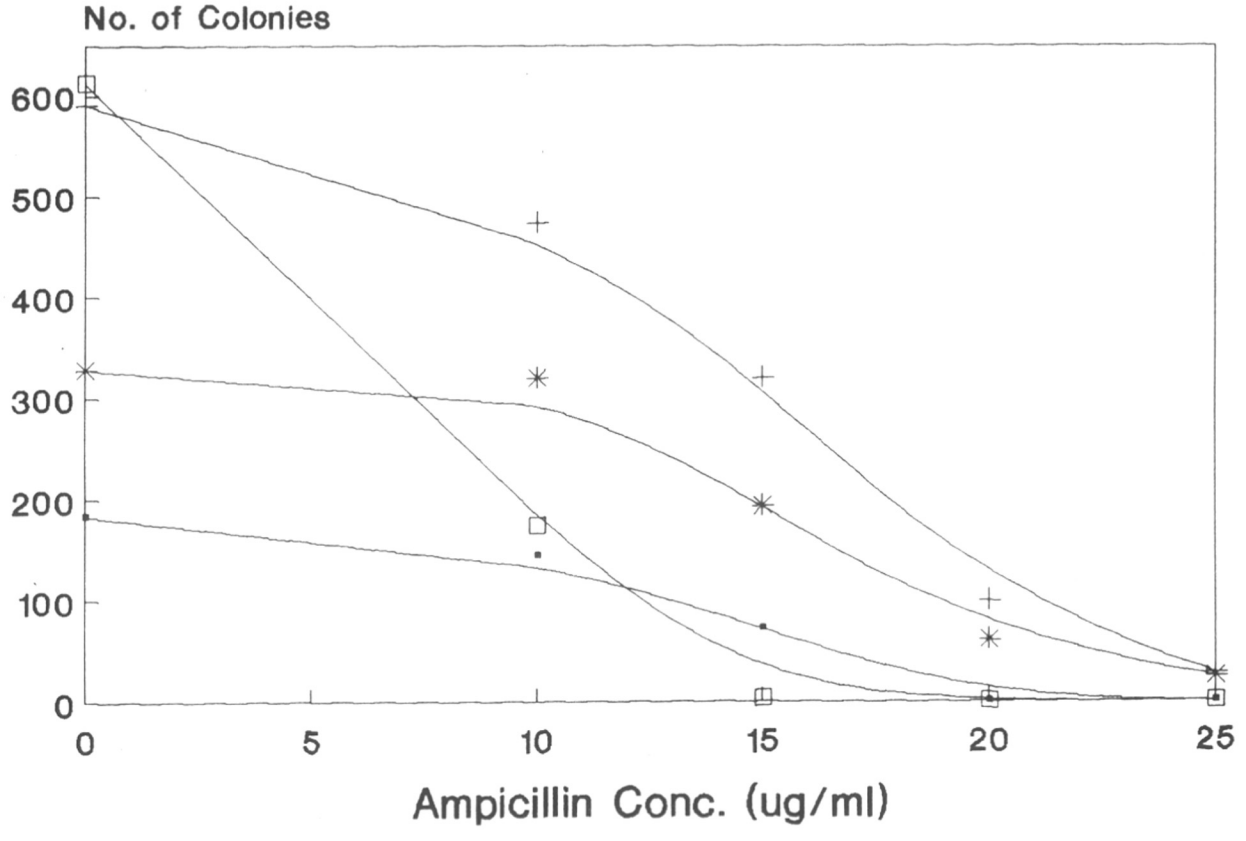
No. of Colonies
600
500
400
300
200
100
0
0
5
10
15
20
25
Ampicillin Conc. (ug/ml)

FIGURE 4.4

Nitrocefin degradation by PCP cell extracts

The change of absorbance was plotted after 1min reaction of nitrocefin with PCP cell extracts.

TABLE 4.1

EXPRESSION OF CHAINIA PROMOTERS IN ESCHERICHIA COLI.

PROMOTERS	LD_{50} (μg/ml amp)	β-LACTAMASE ACTIVITY (Units/ml)
E. coli DH1 (pJAC4)	2-3	0.012
pCP3	13	0.116
pCP4	15	0.145
pCP6	16	0.188
pCP9	7	0.079

DISCUSSION

Our results show that at least a few Chainia promoters are expressed in E. coli. Although it is desirable to selfclone Chainia genes, E. coli still remains the host of choice due to the obvious ease and availability of well standardized techniques for cloning in E. coli. Since Chainia shares some of the genetic characters with Streptomyces, it is likely that, as in case of Streptomyces, it may also possess two types of promoters viz, (i) typical promoters showing homology with prokaryotic consensus sequences and (ii) atypical promoters showing little or no homology with E. coli promoter sequences (13,14).

SEPs (9), HEPS (15,16), and some other streptomycetes promoters such as XP55, pIJ101A,B,C etc. (17,18,19,20) are shown to be expressed in E. coli. However, in spite of the homology with typical prokaryotic consensus sequence, unusual spacing between -10 and -35 and high G+C contents in the spacer sequence of Streptomyces promoters may contribute to their failure of expression in E. coli (21). As shown in the case of gylP1, in addition to -35 and -10, the mutations in spacer sequences too effect promoter strength (22).

The objective of the present studies was to ascertain whether Chainia possesses promoters which are functional in E. coli. Our results show that E. coli

recognizes at least a few Chainia promoters. As evident from the LD_{50} values and β-lactamase activity, these promoters are of varying strengths. While pCP3, 4, and 6 are relatively stronger, pCP9 is the weak promoter. However, the strength of pCP9 parallels with the promoter activity of the wild type ampC gene as revealed by their equal LD50 value (23). Expression of PCPs in E.coli suggests that they resemble SEPs (Streptomyces-E. coli type promoters) isolated by Jaurin and Cohen (9) and belong to the class of typical promoters. Though these results give only general information regarding Chainia promoters, they provide basis for expression of the desired genes from Chainia under the control of strong promoters in E. coli. Streptomyces is shown to have at least one form of RNA polymerase holoenzyme (Er^{35}) that recognizes E. coli type promoters (24,25). However, while some SEPs show a weak promoter activity when cloned back into S. lividans (9), SEP8 is expressed at relatively high levels in both the organisms. Moreover, an apparent difference in the promoter specificity between the two Streptomyces species has also been reported (23). Therefore, promoter elements in sclerotia forming organism like Chainia may not be similar to that of S. lividans. Studying the expression of pCPs in S. lividans can throw some light on these aspects.

Isolation of Chainia promoters also provides a tool

to construct shuttle recombinant plasmids to study the expression of the desired gene/s in *E. coli*, *S. lividans* and probably in *Chainia* too. This approach will be useful particularly for studying the regulatory aspects in the synthesis of many commercially important enzymes that this strain is known to produce.

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Chapter V

DNA Double Helix Stability

Of *Streptomyces* Promoter Sequences

ABSTRACT

In Escherichia coli, the localized unwinding of DNA at -10 region is facilitated by the high free energy content of that region, which, in turn, is due to high frequency of dinucleotides AT and TA in TATAAT box and neighbouring sequences. In the present study, twenty Streptomyces promoter sequences have been analyzed for free energy content of -150 to +50 region using sliding blocks of 12 nucleotides and of -50 to +30 region using a sliding blocks of 6 nucleotides. The results show that Streptomyces -10 region shows high free energy content although this region shows very little homology with TATAAT box. However, in case of Streptomyces, the AT and TA dinucleotides are not the predominant nucleotides in - 10 region. The combined frequency of AT,TA,TT and AA is lower than GG,GC,CG and CC at -10. However, the frequency of the dinucleotide pairs having high free energy (AG,AC,CA,GA,TG,TC,CT and GT) is quite high at -10. It is, therefore, concluded that in Streptomyces, the high free energy at -10 is maintained by the appropriate combination of A or T with G or C.

INTRODUCTION

The extremely G+C rich composition of Chainia and Streptomyces genomes in contrast to the A+T rich nature of typical promoter sequences raises interesting questions about the nature of promoters in Streptomyces. In addition, as in B. subtilis (1), Streptomycetes are likely to have transcription initiation as a key mechanism for the regulation of the complex morphological and biochemical differentiation as evident from the presence of sporulation specific sigma factor whiG in S. coelicolor A3(2) (2,3,4).

E. coli RNA polymerase is known to interact with the promoter in two steps. The first step is the formation of an inactive "closed complex" and its activation to an "open complex". The second step involves localized unwinding of DNA double helix ("melting in") in the middle of -10 region. Margalit et al have shown that this localized unwinding is facilitated by higher free energy content of -10 region as compared to the neighboring sequences (5). This follows directly from the unusually high frequency of TA doublet in the promoter regions of E. coli as the free energy of TA is the highest of all dinucleotides (5).

Taking into consideration the high G+C content of Streptomyces and our findings (unpublished data) that Streptomyces promoters are not as A+T rich as E. coli

even in -10 region, it is proposed

a] To analyze the free energy content in Streptomyces promoters and

b] To study the distribution of dinucleotides responsible for the observed free energy pattern.

The observations and conclusions regarding the double helix stability of 20 Streptomyces promoters are discussed in this chapter.

METHODS

CALCULATION OF FREE ENERGY

The method adopted for calculating free energy was essentially similar to that described by Margalit et al (5). A formula used for calculating the transition free energy from double helix to single strand of a DNA sequence of n nucleotides is (6)

$$\Delta G_n = -(\Delta g_{ini} + \Delta g_{sym}) + \sum_{i=1}^{n-1} \Delta g_{i,i+1}$$

where g_{ini} is the initiation free energy (6kcal/mol for a sequence without any G or C and 5kcal/mol otherwise). g_{sym} accounts for the entropic difference between a DNA double strand formed from a self-complementary sequence and a double strand formed from a sequence of that is not self-complementary (RT ln 2kcal/mol and 0kcal/mol respectively). g_{ij} is the transition free energy for dinucleotide of type ij.

The free energy (Kcal/mol) of each dinucleotide pair is as follows (5):

5' / 3'	A	C	G	T
A	-1.9	-1.9	-1.6	-0.9
C	-1.3	-3.1	-3.1	-1.6
G	-1.6	-3.6	-3.1	-1.9
T	-1.5	-1.6	-1.3	-1.9

Each known sequence in a region of -150 to +50 was divided into sliding blocks of 12 nucleotides and the free energy for each block was calculated using indigenous program BIO2. The accuracy of the program was confirmed by calculating the free energy of oligonucleotides for which the values have been reported earlier (5).

Similar program viz. BIO1 was used for the calculation of free energy of the sliding blocks of six nucleotides in a region of -50 to +30.

Twenty published promoter sequences listed in Table 5.1 were analyzed using programs BIO1 and BIO2 in order to calculate the free energy. The values in each block were normalized by the number of entries in that block and the deviation from mean free energy was plotted against the nucleotide position with respect to a transcription start point. The normalization and deviation were carried out using an indigenous program CAL and the graphs were plotted on LOTUS 1-2-3.

Similarly, FREQ12 program was developed for calculation of frequency of dinucleotide pair/s in the overlapping blocks of 12nucleotides. The frequencies were normalized using CAL and plotted against the nucleotide position as above.

TABLE 5.1

LIST OF PROMOTERS STUDIED FOR FREE ENERGY CONTENTS

PROMOTER	-35 region	bp	-10 region	bp[a]	REF
1: amyP1	T T G A C G	19	C A G G C T	3, 5	19
2: amlP	T T G A C C		T A C G G T		28
3: ß-gal	T T G A T T	18	T A G G T	5, 6	15
4: gylP1	T T G A C G	17	G A G A C T	8	30
5: gylP2	T C G A A C	19	T A G A G T	6	30
6: korB	T T G C G C	17	C A G G A T	6	23,26
7: blaU	T T G G A C	18	G A A G G T	6	17
8: galP1	G T G A C A	21 24	T G T T A T T A T G T T	7 4	16,29
9: kilB (pIJ101pA)	T T G C G C	18	C A G A C T	7	23,26,31,32,
10: pIJ101Pc	T T G A C A	17 14	G A T C G C G C T G A T	8 11	33

TABLE CONTINUED

PROMOTER	-35 region	bp	-10 region	bp[a]	REF
11: ssiP1	AAGAGGCG	18	GGCACTT	3	27
12: ssiP2	GTCGAGGTCA	16	CGGCAGCTC	7	27
13: mel	GTTCGCGGGAGT	10	CCGGTAGGC	5	18
14: rph	CAAATCACCTAGGAGAAGGT	11	CATGAT	7	20
15: galP2	GGAACTTTT	19	GCAAGCTGAA	7	16,29
16: aphP1	CGAAAGGCGCGGAA	18	CATGAT	5	22
17: tsrP1	TTGCCGGTCAGGGCAGCCAT	14	TAGGGT	6	21
18: tsrP2	GCTCGACGCAGCCCAGAAAT	14	AATACT	6	21
19: ØC31 P2:	GTAACGCAC	18	TAGCTT	5	25
20: redD (P11)	CGACCGTGT	17	GCCTGG	7	24

RESULTS

Figure 5.1a shows distribution of free energy in - 150 to +50 region of the Streptomyces sequences under study using a sliding block of 12 nucleotides. The occurrence of a free energy peak in the region around -10 is consistent with the results of Margalit et al for E. coli promoters. However, there are two additional peaks in the -40 and -75 regions which are not observed in case of E. coli.

Analysis using a sliding block of 6 nucleotides in the region of -50 to +30 also revealed a similar pattern (Fig 5.1b). However, the peak at -40 region is not very prominent.

We have divided the twenty promoters under study into two categories: 1) **Typical promoters**: showing partial homology with prokaryotic consensus sequences and 2) **Atypical Promoters**: showing no homology with E. coli promoters. In Table 1, sequence No 1 to 10 are typical promoters while 11 to 20 are atypical. Both types of promoters were separately analyzed using a sliding block of six and twelve nucleotides. In case of typical promoters, the free energy distribution is roughly the same as that of total 20 promoters when a block of 12 n was used (Fig 5.1c). When the 6n blocks of typical promoters were analyzed, a broad region of approximately 20 nucleotides showing high free energy in the -10 region

FIGURE 5.1

Distribution of free energies around the transcription start point.

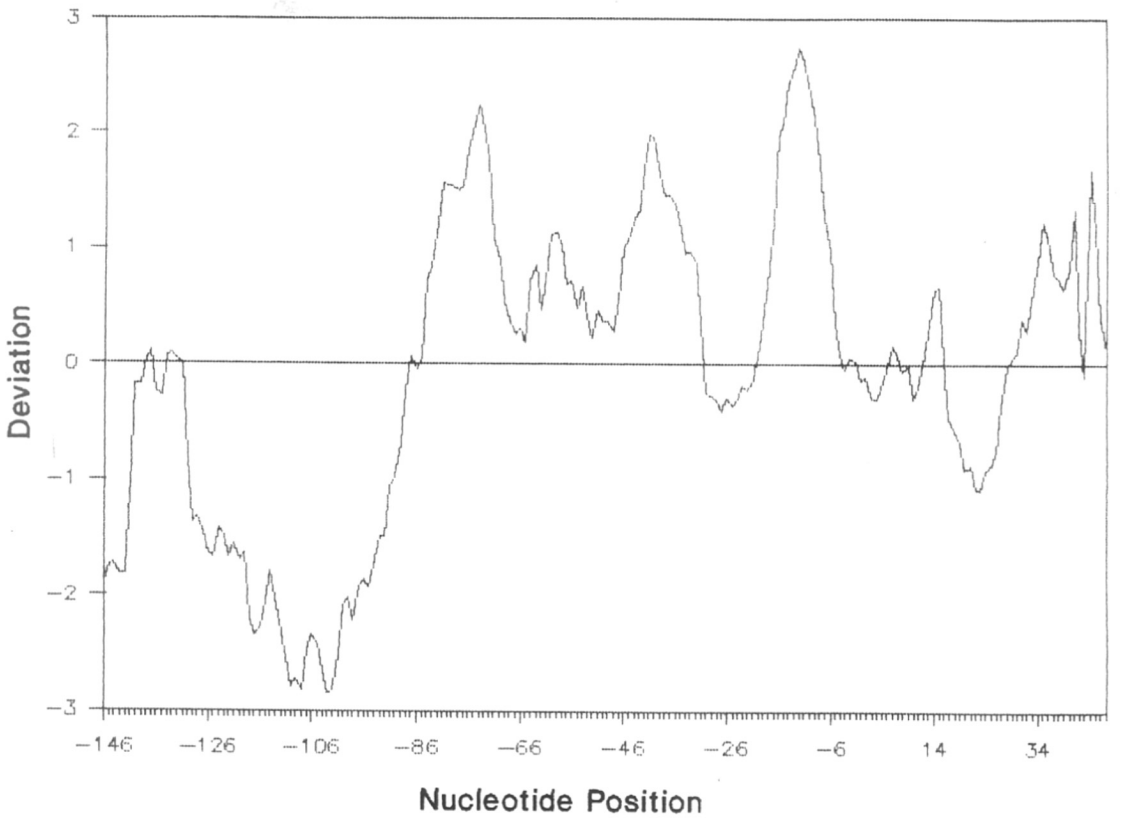
The graphs (Figure 5.1a to Figure 5.1f) show the result of summing and normalizing the dinucleotide free energy values for the Streptomyces promoters listed in Table 5.1. The dashed line represents the mean value of the normalized free energy sum. The position 0 on X-axis is the transcription start point. The nucleotide positions on X-axis represent the mid-point of the sliding blocks of 12n or 6n, as specified in the figures.

FIGURE 5.1a

Distribution of free energy in -150 to +50 region of 20 Streptomyces promoters.

The sliding blocks of 12 nucleotides were used for calculating free energy values.

FIG. 5·1a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2
1
0
−1
−2
−3
Deviation
−146
−126
−106
−86
−66
−46
−26
−6
14
34
Nucleotide Position

FIGURE 5.1b

Distribution of free energy in -50 to +30 region of 20 *Streptomyces* promoters.

The sliding blocks of 6 nucleotides were used for calculating free energy values.

FIG. **5.1** b

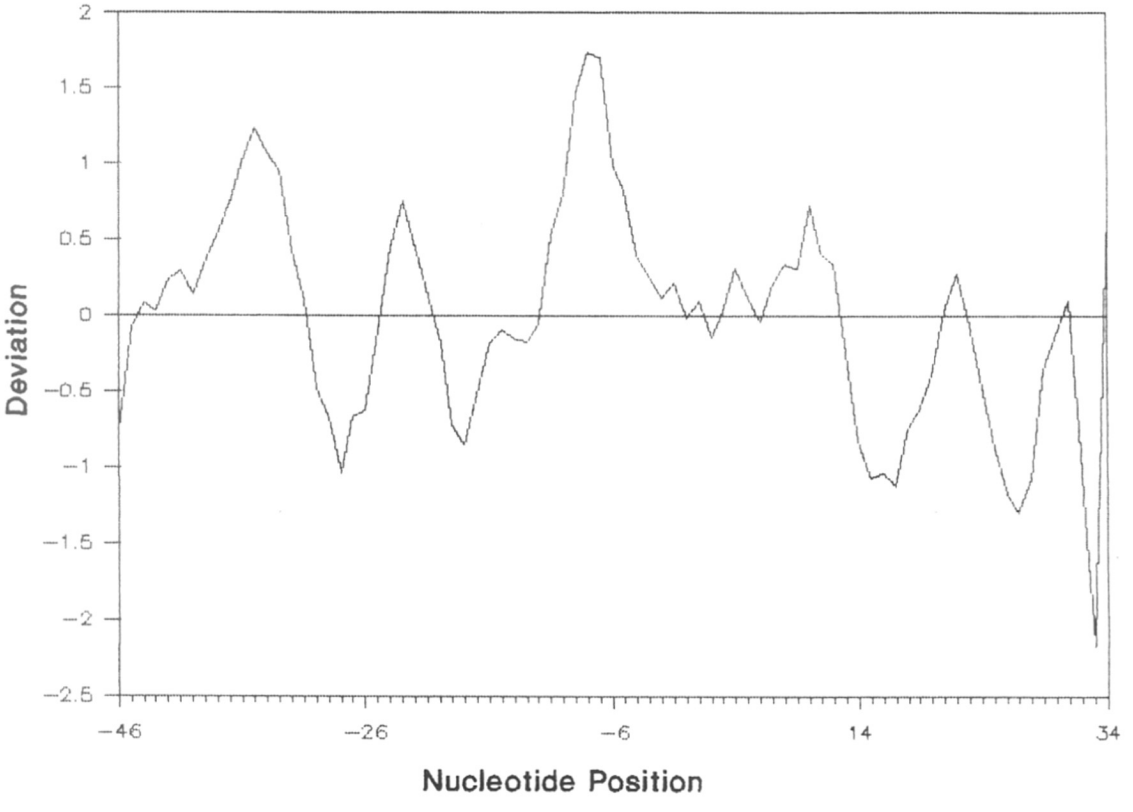
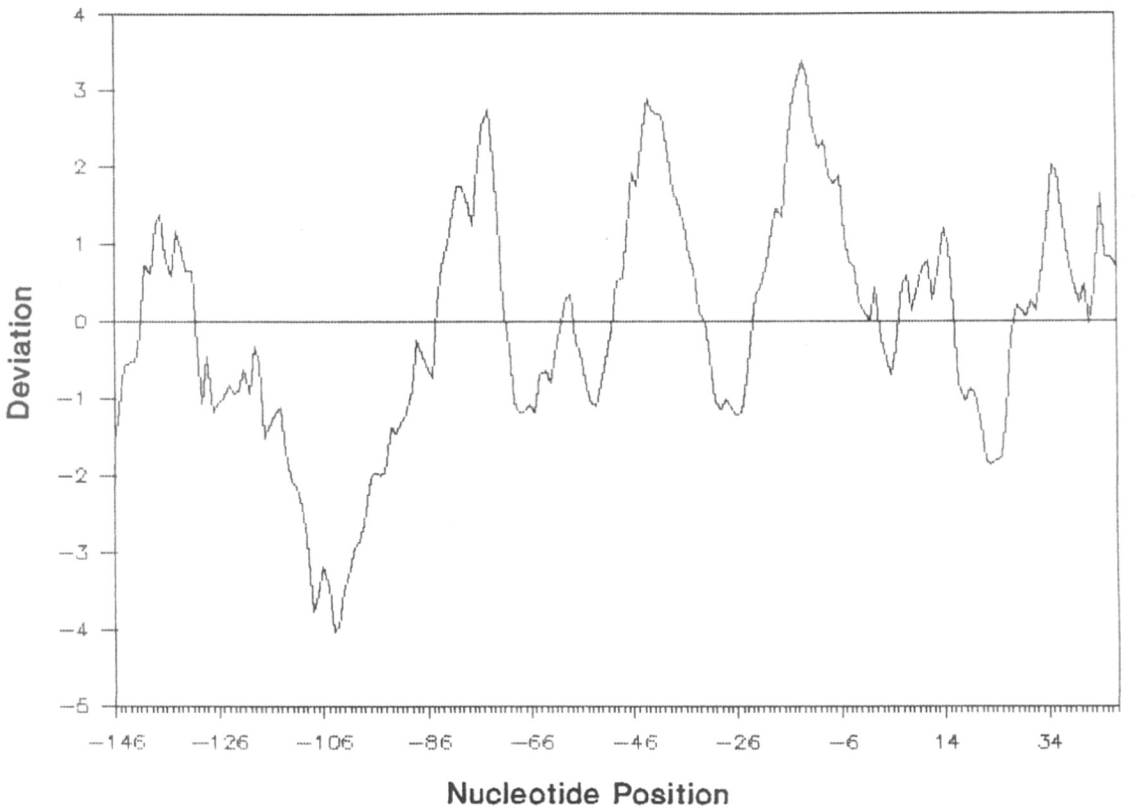

2
1.5
1
0.5
0
−0.5
−1
−1.5
−2
−2.5
Deviation
−46
−26
−6
14
34
Nucleotide Position

FIGURE 5.1c

Distribution of free energy in -150 to +50 region of Typical *Streptomyces* promoters.

The sliding blocks of 12 nucleotides were used for calculating free energy values.

FIG. 5-1c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3
2
1
0
−1
−2
−3
−4
−5
Deviation
−146
−126
−106
−86
−66
−46
−26
−6
14
34
Nucleotide Position

was observed (Fig 5.1d). In addition, there were two peaks at -25 and -40. However, in case of atypical promoters, three peaks; one at -10 and the other two peaks at -40 and -75 were observed (Fig 5.1e). When a block of 6 nucleotides was used, atypical promoters showed a sharp peak at -10 region (Fig 5.1f).

In order to assess the nature of dinucleotides which contribute to the observed free energy, the frequencies of various dinucleotides in the region -150 to +50 were calculated. The following combinations of dinucleotides were analyzed for their frequency of occurrence.

a] AT

b] TA

c] CG

d] AT+AA+TA+TT

e] GC+GG+CG+CC

f] AG+AC+GA+CA+TG+TC+GT+CT

There are striking differences in the frequencies of distribution of these doublets between Streptomyces and E. coli. Frequency of AT doublet is the highest at -10 as compared to its frequency at other positions in a region of -150 to +50, although there is an additional region of high AT frequency at -50 (Fig 5.2). Similarly, The frequency of TA doublet is also highest at -10 with respect to its occurrence at other positions, but considerably lower than that of AT. Though the frequency

FIGURE 5.1d

Distribution of free energy in -50 to +30 region of Typical *Streptomyces* promoters.

The sliding blocks of 6 nucleotides were used for calculating free energy values.

FIG. **5.1**d

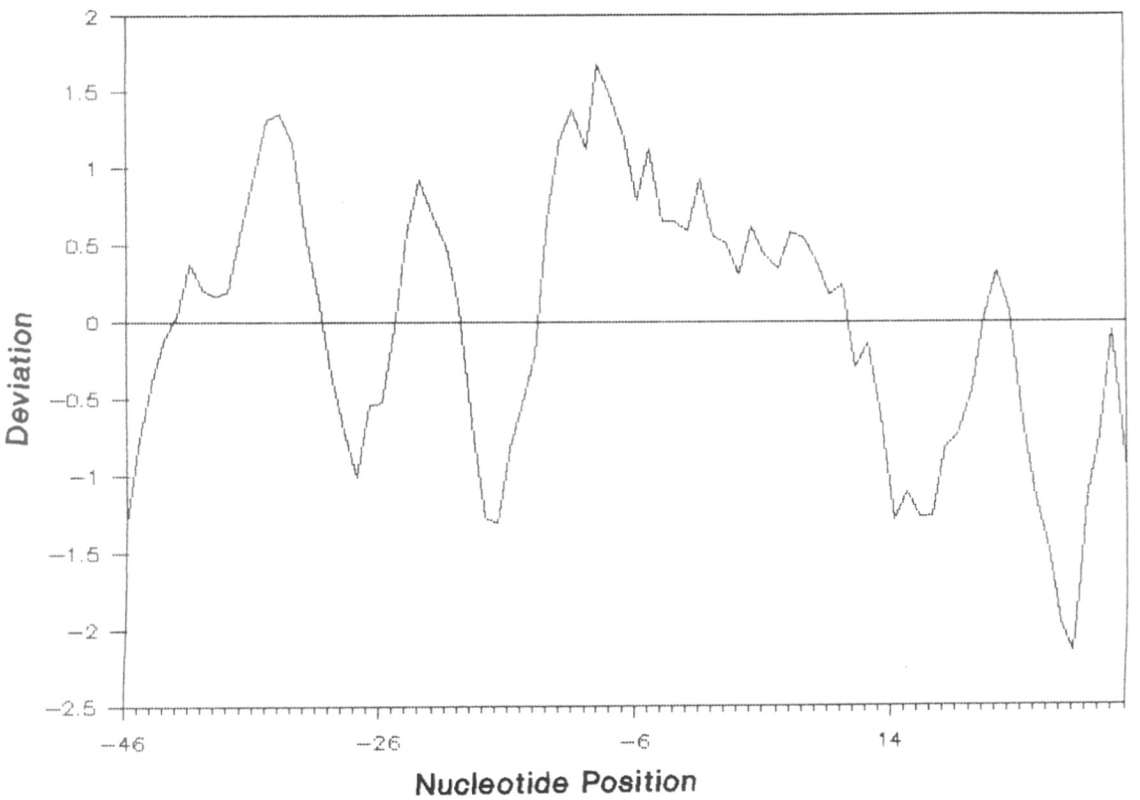


FIGURE 5.1e

Distribution of free energy in -150 to +50 region of Atypical *Streptomyces* promoters.

The sliding blocks of 12 nucleotides were used for calculating free energy values.

FIG. 5-1e

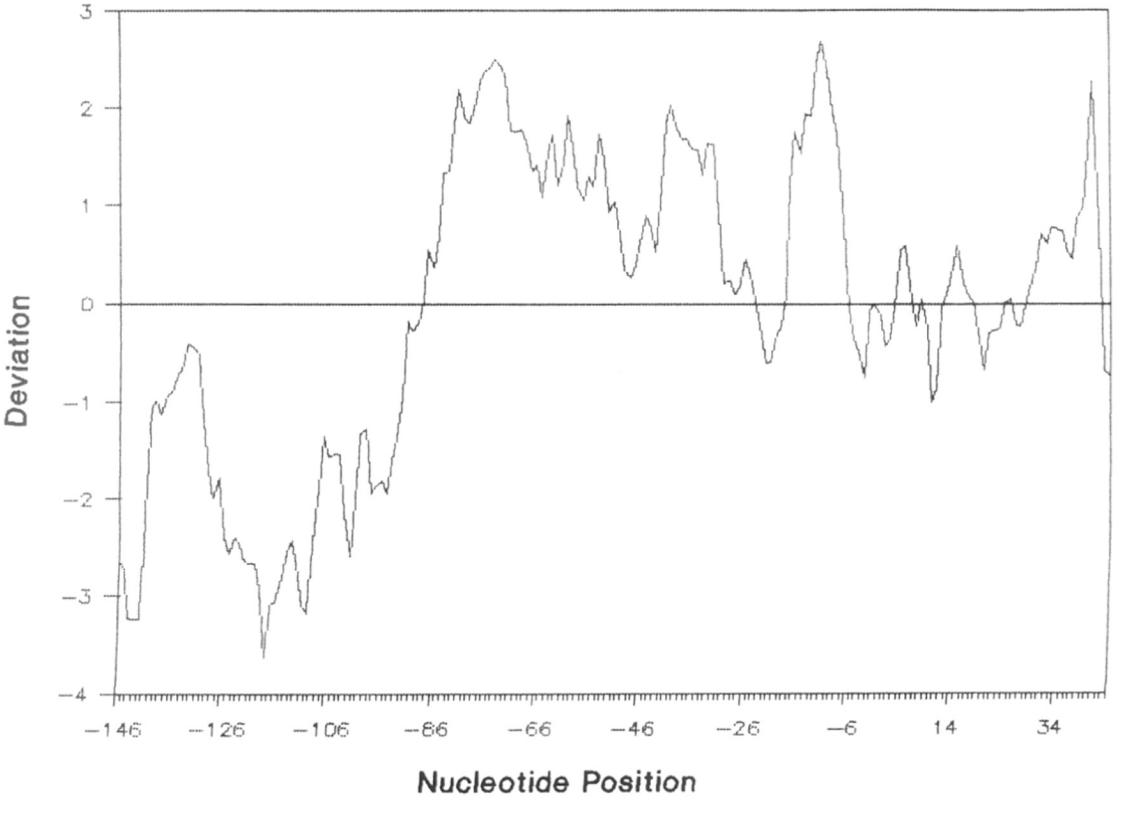

3
2
1
0
−1
−2
−3
−4
Deviation
−146
−126
−106
−86
−66
−46
−26
−6
14
34
Nucleotide Position

FIGURE 5.1f

Distribution of free energy in -50 to +30 region of Atypical Streptomyces promoters.

The sliding blocks of 6 nucleotides were used for calculating free energy values.

FIG. 5.1f

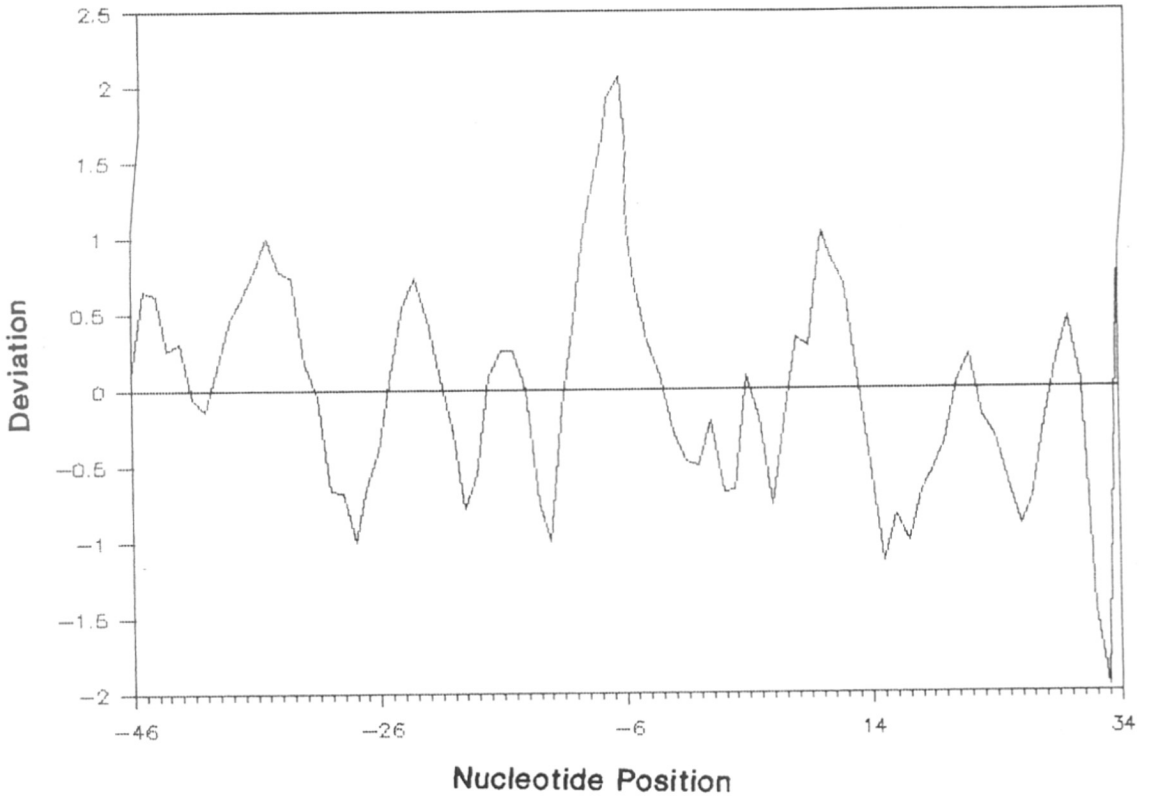

2.5
2
1.5
1
0.5
0
−0.5
−1
−1.5
−2
Deviation
−46
−26
−6
14
34
Nucleotide Position

FIGURE 5.2

Distribution of AT,TA and CG dinucleotides around transcription start points.

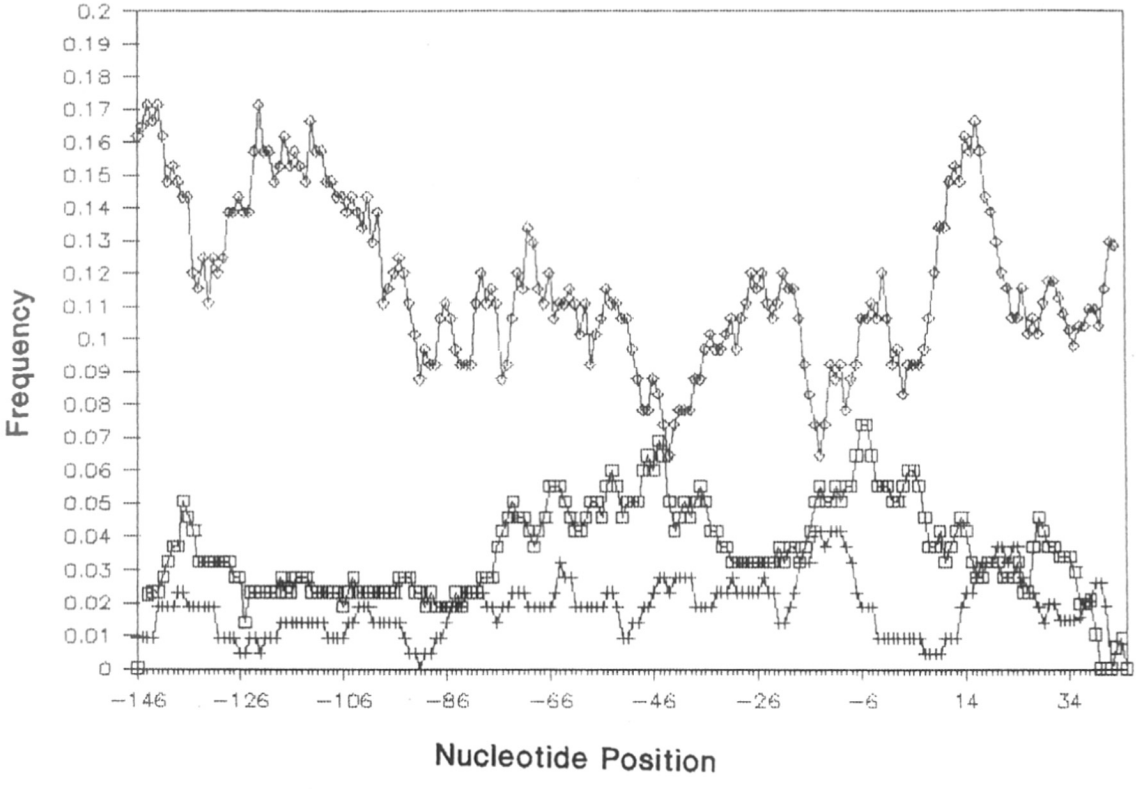
The frequencies of AT,TA and CG in the region -150 to+50 were calculated using the overlapping blocks of 12 nucleotides. The nucleotide positions on X-axis represent the mid-point of each block.

AT —□—□—

TA —+++—

CG —◇—◇—

FIG. 5.2


0.2
0.19
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Frequency
−146
−126
−106
−86
−66
−46
−26
−6
14
34
Nucleotide Position

of AT and TA is higher at -10 as compared to the other positions, they are not the major nucleotide doublets in -10 region as their frequency is only 0.074 and 0.041 respectively. The frequency of CG (0.087) is the highest among AT, TA and CG (Fig 5.2).

The combined frequency of AT,TA,TT and AA shows a major peak at -60 and two other peaks at -40 and -10 respectively (Fig 5.3). On the other hand, combined frequency of GG,GC,CG and CC is the lowest at -10 as compared to the other positions in the -150 to +50 region. However, even at -10, it is higher than the combined frequency of AT,TA,TT and AA (0.27 and 0.19 respectively). It is evident from the figure that at no position in a region of -150 to +50, the combined frequency of AT,TA,TT,AA is higher than GG,GC,CG and CC though the individual doublets in the latter group have lower free energy content (-3.1kcal/mol to - 3.6kcal/mol) as compared to that of the former group (-0.9kcal/mol to -1.9 kcal/mol). Therefore it appears that AT,TA,TT and AA do not contribute significantly to the observed high free energy content of -10 region.

The combined frequency of dinucleotides containing A or T with G or C (viz. AG,AC,GA,CA,TG,TC,GT,CT) is maximum at -10 (Fig 5.4). It is important to note that the free energy values of these 8 pairs lie in the range of -1.3 kcal/mol to -1.9kcal/mol as that of AA,AT and TT.

FIGURE 5.3

Distribution of AT,TA,TT,AA and GG,GC,CG,CC dinucleotide pairs around transcription start points.

The combined frequencies of AT,TA,TT,AA and GG,GC,CG,CC in the region -150 to+50 were calculated using the overlapping blocks of 12 nucleotides. The nucleotide positions on X-axis represent the mid-point of each block.

AA,AT,TT, TA

GG, GC, CG, CC

FIG. 5-3

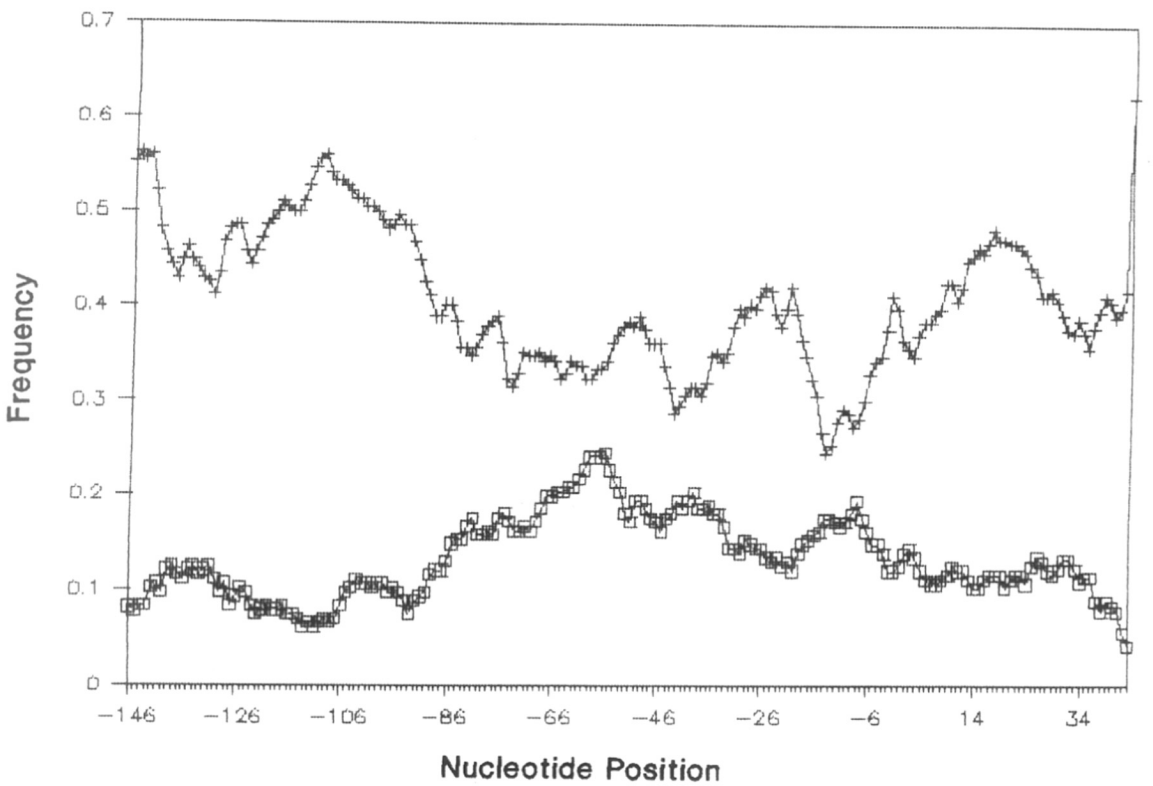
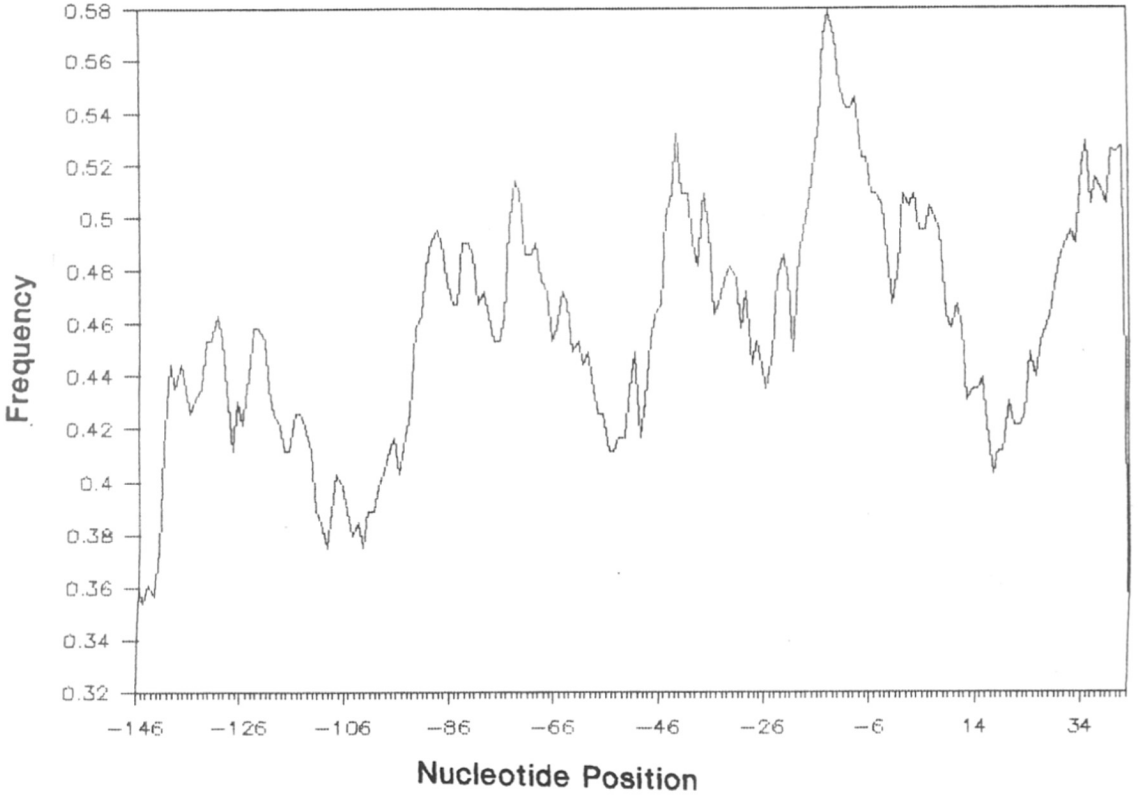

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Frequency
−146
−126
−106
−86
−66
−46
−26
−6
14
34
Nucleotide Position

FIGURE 5.4

Distribution of AG,TG,TC,TG,AC,AG,CA,GA dinucleotide pairs around transcription start points.

The combined frequency of AG,TG,TC,TG,AC,AG,CA,GA in the region -150 to+50 was calculated using the overlapping blocks of 12 nucleotides. The nucleotide positions on X-axis represent the mid-point of each block.

FIG. 5.4


0.58
0.56
0.54
0.52
0.5
0.48
0.46
0.44
0.42
0.4
0.38
0.36
0.34
0.32
Frequency
−146
−126
−106
−86
−66
−46
−26
−6
14
34
Nucleotide Position

DISCUSSION

The results of this analysis show that the -10 region of Streptomyces genes has relatively high free energy contents. This observation is analogous to that made for E. coli promoters by Margalit et al. In E. coli, the high free energy at -10 is correlated with the preponderance of AT and TA doublets which have high free energy values (-1.5kcal/mol and -0.9kcal/mol respectively). However, the corresponding high frequency of AT and TA doublets is not observed in Streptomyces. Our results on the distribution of various dinucleotides in Streptomyces show that the high free energy content in -10 is attained by the appropriate combination of G or C with A or T (Fig 5.3). This observation is reasonable in view of the G+C rich nature of Streptomyces genome and compromises for the significantly lower free energy values of GG,GC,CG,CC doublets.

Out of the sixteen dinucleotide pairs, only four (GG,GC,CG,CC) have considerably lower free energy content. The combined frequency of these four doublets is relatively lower in -10 region. The four doublets AT,TA,TT,AA have high free energy content but their frequency is not high at -10. However, the combined frequency of AG,AC,GA,CA,TG,TC,GT,CT is significantly higher in the same region. These eight doublets, together with AT,TA,TT,AA, constitute more than 75% of the doublets in the -10 region, of which the contribution of

AT,TA,TT,AA is quite low (19%). This is consistent with our finding that very few Streptomyces -10 regions are A+T rich. Even in case of typical promoters, there is no significant homology with prokaryotic consensus sequence in -10 region, except A at second position and T at 6th position in the TATAAT box. The homology is more prominent in -35 region (Table 5.2), (7).

In case of E. coli, it was shown that in 40 out of 50 down mutations, there is a decrease in free energy content and 77% of up mutations are less stable as compared to wild type (5). However, there are no reports of mutations in -10 region of Streptomyces except in case of aphP1 (22), where it was found that mutations increasing the homolgy with typical E. coli promoters have higher strength as compared to wild type.

Though free energy content of -10 region seems to be an important factor in transcription initiation, there are several other factors that may influence the process. In case of Streptomyces, the heterogeneity of RNA polymerases plays a crucial role in gene regulation and differentiation (8,9). Though the property of localized unstable region might appear in all types of promoter, the presence of four sigma factors (hrdA, hrdB, hrdC and hrdD) similar to σ^{70} of E. coli may enable to recognize the subtle differences in promoter sequences (10,11,12). Margalit et al did not divide the promoters according to

their RNA polymerase specificity while studying E. coli promoters. In the present study, the classification of typical and atypical promoters was made only from the point of view of pinpointing the subtle differences in the two types of promoters. It should be noted that atypical promoters is not a class by itself as it comprises of the sequences that might be recognized by totally different forms of RNA polymerases. Even in case of typical promoters, not all have been experimentally shown to be recognized by the RNA polymerase holoenzyme homologous to σ^{70} of E. coli. The results show that the unstable -10 region is a global phenomenon irrespective of sigma factor specificity.

The significance of other regions of high free energy content in Streptomyces remains obscure. According to bipartite model, -35 region is involved only in the recognition while -10 region is responsible for open complex formation (13). The validity of this model is questioned by Margalit et al who have revealed that 20% of mutations in -10 region do not show correlation between the direction of free energy change and the type of mutation. This has been attributed to the effect of mutation on appropriate positioning of the enzyme rather than double helix stability. In case of galP11 of S. coelicolor, the mutations in -18 and -20 shows significant effect on the strength of the promoter, which was thought to be due to the conformational changes in

DNA structure (30).

It is also possible that besides containing common sequences, promoters may contain common structural features that would not be revealed by searches based on sequence homology. The two regions may possess similar structures although their sequences are not homologous. Chang-Shung Tung observed that particular patterns of helix twist angles are present in promoters with a high frequency (14). The detailed studies on these lines, in addition to extensive data on the effect of mutations on promoter strengths in Streptomyces is likely to reveal some novel features of Streptomyces promoters.

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