Characterization of Plasmids from Natural Isolates of Lactic Acid Bacteria: Analysis, Identification and Screening for Novel Promoters

THESIS

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By

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CERTIFICATE

This is to certify that the thesis entitled, **"Characterization of Plasmids from Natural Isolates of Lactic Acid Bacteria: Analysis, Identification and Screening for Novel Promoters"** which is submitted by Mr. Narayan Kumar ID. NO. 2004PHXF421 for the award of Ph.D. degree of the institute embodies original work done by him under my supervision.

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Dedicated

to

my parents, my wife & my kid

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ABSTRACT

Isolation and characterization of plasmid DNA from lactic acid bacteria and their promoter screening and analysis are important for applications related areas, which have implications for biotechnology based industries. Twenty one Lactic acid bacteria were isolated from milk, water, soil and plant samples by culturing the samples on SSMD and MRS media. They were identified at genus level by different biochemical and physiological tests. Out of the 21 isolates, 10 were lactobacilli, 3 were enterococci, 2 were staphylococci, 5 were lactococci and 1 was *Leuconostoc*. These isolates were then screened for the presence of plasmids. Out of these isolates screened, only six strains were found to harbor plasmids and they were identified at species level by 16S rRNA gene amplification and sequencing. Further, these isolates were screened for antibiotic susceptibility, antimicrobial activity against pathogenic microbes and carbohydrate fermentation. Limited studies have been performed on the characterization of small size plasmids of *Enterococcus faecium* with the intention of evaluating the strength of their promoters in *E.coli*. The complete nucleotide sequence (3.825 Kb) and structural organization of *Enterococcus faecium* DJ1 cryptic plasmid pNJAKD was determined. Analysis of this plasmid demonstrated that it generates single-stranded DNA intermediates, and sequence analysis revealed that it contains five putative open reading frames (ORFs). Two major open reading frames (ORF4 & ORF5) with a good coding probability are located on the same strand and cover 80% of the total sequence. The larger open reading frame (ORF4) encodes a putative polypeptide which exhibits sequence similarity with the Recombinase (Mob) protein of number of Gram-positive plasmids including pJS42 and pRI1 from *Enterococcus faecium*. ORF5 encodes a putative polypeptide which exhibits sequence similarity with the known replication proteins of RCR replicons, particularly those of the pC194 family. Seven promoter sequences from the pNJAKD plasmid of *Enterococcus faecium* were identified. The regions coding for the putative promoters were either amplified using PCR based techniques or chemically synthesized as oligonucleotides of different sizes. These were subsequently cloned in the pEGFP vector at the *Pvu* II site. The efficiency of putative promoter fragments were measured using the intensity of eGFP fluorescence in *Escherichia coli* JM101, DH5α and BL21(DE3), among

which AKD3 exhibited moderate to strongest promoter activity at temperatures of 30, 37, and 42 °C.

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Abbreviations

LAB	Lactic acid bacteria
GRAS	Generally recognized as safe
sso	Single-strand origin
ssoA	Single-strand origin A-type
ssoU	Single-strand origin U-type
ssoT	Single-strand origin T-type
ssoW	Single-strand origin W-type
dso	Double-strand origin
RCR	Rolling circle replication
oriT	Origin of transfer
ori	Origin of replication
eGFP	Efficient green fluorescent protein
aa	Amino acids
r DNA	Ribosomal DNA
r RNA	Ribosomal RNA
IP	Isoelectric point
EDTA	Ethylene diamine tetra acetate
Tris	Tris(hydroxylmethyl)aminomethane
SDS	Sodium dodecyl sulphate
q.s.	Quantity sufficient
kDa	Kilo dalton
PAGE	Poly acryl amide gel electrophoresis
mM	Mili molar
M	Molar
µM	Micro molar
min	Minute
s	Second
kbp	Kilo base pair
bp	Base pair
MW	Molecular weight
ORF	Open reading frame

Mob	Mobilization protein
Rep	Replication protein
IPTG	Isopropyl - β - D- thiogalactoside
h	Hour
BCP	Bromo cresol purple
SSMD	Sodium Acetate Skim Milk Dextrose
LB	Luria-Bertani
MRS	deMann and Rogosa
SD	Standard deviation
HSP	Heat shock protein
RS_B	Recombination site
DNA Pol	T4 DNA polymerase
PNK	T4 Phospho nucleotid kinase
rpm	Rotation per min
IR	Inverted repeats
dNTPs	Deoxy ribonucleoside triphosphates
nt	Nucleotide

Amino acid	One letter symbol	Three letter symbol
Glycine	G	gly
Valine	V	val
Leucine	L	leu
Isoleucine	I	ile
Alanine	A	ala
Arginine	R	arg
Asparagine	N	asn
Aspartic acid	D	asp
Cysteine	C	cys
Glutamine	Q	gln
Glutamic acid	E	glu
Histidine	H	his
Lysine	K	lys
Methionine	M	met
Phenyl alanine	F	phe
Proline	P	pro
Serine	S	ser
Threonine	T	thr
Tryptophan	W	trp
Tyrosine	Y	tyr

Nucleotide bases

Adenine	A
Guanine	G
Cytosine	C
Thymine	T
Uracil	U

Chapter 1

Lactic acid bacteria Plasmids and Promoters - An introduction

Chapter 1

1. INTRODUCTION

Biotechnology industries continuously need new expression vectors. Expression vectors are effective tools for optimization of high level of protein production in the cell (Kim and Mills, 2007). The wealth of naturally occurring plasmids in the lactic acid bacteria could provide infinite opportunities to genetic engineers for construction of cloning and expression vectors. There are several expression and cloning vectors available based on lactic acid bacteria plasmids. However, researchers still have just a few broad host range cloning and expression vectors. There have been limited reports suggesting that promiscuous plasmids exist in the Gram-positve (+ve) lactic acid bacteria (LAB) which can also propagate in Gram-negative (–ve) organisms (del Solar *et al.*, 1993; Platteeuw *et al.*, 1994). We were interested in identifying promoters which would be efficient in both Gram +ve and Gram –ve organisms so as to enable creation of novel vectors with multiple use.

This review focuses on the following aspects

1.1. Lactic acid bacteria

1.2. Genera of lactic acid bacteria

1.3. Lactic acid bacteria plasmid

- **1.3.1.** RCR replicating plasmids
- **1.3.2.** Theta-replicating plasmids
- **1.3.3.** Conjugative and Mobilizable plasmids (Promiscuous plasmids)

1.4. Enterococcal plasmids

- **1.4.1.** Broad host range RCR plasmid
- **1.4.2.** Inc 18 plasmids
- **1.4.3.** Pheromone responsive plasmids

1.5. Vector derived from lactic acid bacteria plasmid

- **1.5.1.** Enterococcal cloning and expression vectors

1.6. Controlled expression systems

- **1.6.1.** Sugar inducible expression system
- **1.6.2.** Expression based on phage promoters
- **1.6.3.** Acid inducible expression systems
- **1.6.4.** Nisin controlled expression system

1.1. Lactic acid bacteria

Lactic acid bacteria constitute a Gram-positive, none spore forming, cocci, coccobacilli or rod shaped bacteria, which include, lactobacillus, Leuconostoc, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Teragenococcus*, *Vagococcus*, and *Weisella* (Todar, 2009). Lactic acid bacteria are usually catalase-negative bacteria, which grow under microaerophilic to strictly anaerobic conditions (Talwalkar and Kailasapathy, 2004). Although, they lack catalase, they possess superoxide dismutase and have alternative means of detoxifying peroxide radicals generally through peroxidase enzymes (Wang *et al.*, 2009). They have limited biosynthetic ability, having evolved in environments that are rich in amino acids, vitamins, purines and pyrimidines (Cogan *et al.*, 1997). Therefore, they must be cultivated in complex media that provides all the nutritional requirements. They are found on decaying plant materials, gastrointestinal tract of vertebrates, in sewage, milk and milk products and soil (Chen *et al.*, 2005; Musikasang *et al.*, 2009).

Lactic acid bacteria are among the most important groups of microorganisms used in food fermentations. They contribute to the taste and texture of fermented products and inhibit food spoilage bacteria by producing growth-inhibiting substances and large amounts of lactic acid (Table 1.1). As agents of fermentation LAB are involved in making yogurt, cheese, cultured butter, sour cream, sausage, cucumber pickles, olives and sauerkraut, but some species may spoil beer, wine and processed meats (Geis, 2003). Their traditional use in the food industry confirms their lack of pathogenicity; therefore, they are recognized as safe (GRAS) organisms (Axelson, 1998; Stiles and Holzapfel, 1997).

Table 1.1: Growth inhibiting substances and their mode of action

Growth Inhibiting Substance	**Mode of action**
Carbondioxide	Inhibit decarboxylation Reduces membrane permeability
Diacetyl	Interacts with arginine-binding proteins
Hydrogen Peroxide	Oxidizes basic proteins
Lactic acid	Undissociated lactic acid penetrates the membranes, lowering the intracellular pH. It also interferes with metabolic processes such as oxidative phosphorylation
Bacteriocin	Affect membranes, DNA-synthesis and protein synthesis.

1.2. The genera of Lactic acid bacteria

1.2.1. *Streptococcus*

Streptococci are oxidase-negative, catalase-negative, facultative anaerobic, Gram-positive bacteria. They are often found in pairs or chains form (Stiles and Holzapfel, 1997). In 1984, many organisms formerly considered *Streptococcus* were separated out into the genera *Enterococcus* and *Lactococcus* (Facklam, 2002).

Certain *Streptococcus* species are responsible for meningitis, bacterial pneumonia, and endocarditis However, many streptococcal species are nonpathogenic, and form part of the commensal human microhabitat of the mouth, skin, intestine, and upper respiratory tract.

Streptococcus species are classified based on their hemolytic properties (Parija, 2009)

Alpha-hemolytic species: Species cause oxidization of iron in hemoglobin molecules within red blood cells, giving it a greenish color on blood agar. Their representative species are S. pneumoniae, and Streptococcus viridians group

Beta-hemolytic species: Species cause complete rupture of red blood cells. On blood agar, this appears as wide areas clear of blood cells surrounding bacterial colonies. Representative species include, S. pyrogenes, S. agalactiae

Gamma-hemolytic species: Species cause no hemolysis. This includes the species of genus Enterococcus, which is now separated from the streptococcus genus.

1.2.2. *Enterococcus*:

Enterococci are Gram-positive, facultative anaerobic and cocci bacteria. They occur singly, in pairs or as short chains. They can be found in soil, water, food, animals and humans (Stiles and Holzapfel, 1997). Enterococci are able to tolerate a wide range of environmental conditions: extreme temperature (10-45°C), pH (4.5-10.0) and high sodium chloride concentrations (Fisher and Phillips, 2009). The genus *Enterococcus* harbors many different species, but four of them are the most common species in farm animals and humans. They are *E. faecium*, *E. faecalis*, *E. durans*, and *E. hirae*. Most Enterococci strains cause a range of infections in the community setting, such as endocarditis, pelvic infections, neonatal infections and urinary tract infections (Arias and Murray, 2012).

1.2.3. Lactococcus

Lactococci are Gram-Positive, catalase-nagative, non-motile cocci bacteria. They are characterized by ovoid cells which appear individually, in pairs, or in chains. They grow at a temperature of 10 °C, but not at 45 °C (Stiles and Holzapfel, 1997). They are known as homofermentors, because they produce a single product, lactic acid in this case, as the major or only product of glucose fermentation. Their homofermentative nature can be altered by adjusting culture conditions like pH, nutrient limitation and glucose concentration. The lactococci comprise the species L. lactis, L. garvieae, L. raffinolactis, L. plantarum, L. piscium, L. fuiiensis, and L. chungangensis (Stiles and Holzapfel, 1997).

In particular, there is a special interest on the study of L.lactis, because they are used as starter culture in industrial dairy fermentations (Stiles and Holzapfel, 1997).

The bacteria also play a role in the flavor of the final product. Lactococcus lactis became famous as the first genetically modified organism to be used alive for the treatment of human disease (Braat *et. al.*, 2006).

1.2.4. Lactobacillus

Lactobacilli are rod-shaped, Gram-positive, fermentative, organotrophs. They are usually straight, although they can form spiral or coccobacillary forms under certain conditions. They are often found in pairs or chains of varying length. Most species of lactobacilli are homofermentative, but some are heterofermentative. The genus has been divided into three major subgroups based on fermentation profile (Stiles and Holzapfel, 1997): Group I: lactobacilli are obligately homofermentative and produce lactic acid as a major end product (>85%) from glucose. They grow at 45 °C, but not at 15 °C. Representative species include *L. delbrueckii* and *L. acidophilus*. Group II: lactobacilli are homofermentative, but they grow at 15 °C and show variable growth at 45 °C. Their representative species are *L. casei* and *L. plantarum*. Group III: lactobacilli are heterofermentative. Aldolase is absent and phosphoketolase is present. They are represented by *L. fermentum, L. brevis* and *L. keferi*. The lactobacilli are more resistant to acidic condition (able to grow at pH values as low as 4 (Todar, 2009)

1.2.5. Leuconostoc

Leuconostoc are catalase-negative, non-sporulating, facultatively anaerobic, Gram-positive bacteria. All the species of leuconostoc are heterofermentative, produces D-lactate, ethanol and CO_2 by glucose fermentation. Leuconostoc are usually spherical cells, sometimes rather lenticular, and resemble very short bacilli with rounded ends. The cells are arranged in pairs or chains. In nutrient media during the active growth phase, they are often in short chains, whereas in their natural environment and more stressful conditions the chains are longer. Most of these bacteria grow at 10 to 40 °C, but not at 45 °C (Bjkroth and Holzapfel, 2006)

1.3. Lactic acid bacteria plasmids

There are a large number of cryptic plasmids which have been reported in the lactic acid bacteria. The sizes range from 1 to >100 Kb (Shareck *et al.*, 2004). Linear plasmids have been also found in lactic acid bacteria and their structure can be of two types; those having a hairpin at each end, and those having a protein covalently bound at their 5′ end (Salminen *et al.*, 2011).

Lactic acid bacteria plasmids are grouped into three categories based on their replication and conjugation functions

1.3.1. Rolling circle replicating plasmids:

1.3.2. Theta replicating plasmids

1.3.3. Conjugative and Mobilizable Plasmids

1.3.1. Rolling circle replicating plasmids

Rolling-circle replicating plasmids are common in Gram-positive bacteria, also found in Gram-negative bacteria and in archea (Khan, 1997). Most of the rolling-circle replicating plasmids are small size (smaller than 10Kb) cryptic plasmids (Khan, 2005). However some of the RCR plasmids carry genes for mobilization and transfer (Lorenz-Diaz and Espinosa, 2009). One of the best features of many RCR plasmids is that broad host range capacity. All RCR plasmids of Gram-positive bacteria belong to at least five major families (pT181, pC194, pMV158, pSN2, pTX14-3) (Table 1.2) (Khan, 1997; Shareck *et al.*, 2004). Individual family members share homology in

their origin of replication and gene encoding the initiator protein. RCR plasmid contains rep gene that encodes the replication initiation protein (Rep) controlled by repressor and its target site, double-stranded origin (*dso*). Additionally most RCR plasmids have a single-stranded origin, for the conversion of SS-DNA intermediates into DS-DNA. The steps of rolling-circle mechanism and genetic elements that are involved in rolling-circle replication are described below.

Table 1.2: RC plasmids of Gram-positive bacteria (Khan 1997; Shareck *et al.*, 2004)

Plasmid	Size (Kb)	Original host
pT181		
pT181	4.4	*Staphylococcus aureus*
pC221	4.6	*Staphylococcus aureus*
pC223	4.6	*Staphylococcus aureus*
pCW7	4.2	*Staphylococcus aureus*
pUB112	4.1	*Staphylococcus aureus*
pT127	4.4	*Staphylococcus aureus*
pS194	4.4	*Staphylococcus aureus*
pOg32	2.5	*Leuconostoc oenos*
pHD2	2.1	*Bacillus thuringiensis*
pRS1	2.5	*Oenococcus oeni*
pFR18	1.8	*Leuconostoc mesenteroides*
pC194		
pC194	2.9	*Staphylococcus aureus*
pAMα1	9.6	*Enterococcus faecalis*
pBAA1	6.8	*Bacillus subtilis*
pUB110	4.5	*Staphylococcus aureus*
pBC16	4.6	*Bacillus cereus*
pBC1	1.6	*Bacillus coagulans*
pVA380-1	4.2	*Streptococcus ferus*
pOX6	3.2	*Staphylococcus aureus*
pLP1	2.1	*Lactobacillus plantarum*
pFTB14	8.2	*Bacillus liquefaciens*
pC30il	2.1	*Lactobacillus plantarum*
pBS2	2.3	*Bacillus subtilis*
pBP614	5.6	*Bacillus popilliae*
pSN1981	4.9	*Bacillus subtilis*
pLo13	3.9	*Leuconostoc oenos*
pTB19	1.75	*Bacilli*
pWC1	2.8	*Lactococcus lactis*
pUH1	5.7	*Bacillus subtilis*
pTA1060	8.6	*Bacillus subtilis*
p353-2	2.4	*Lactobacillus pentosus*
pTC82	7.0	*Latobacillus reuteri*
pLAB1000	3.3	*Latobacillus hilgardii*
pST1	2.1	*Streptococcus thermophillus*

pER8	2.2	*Streptococcus thermophillus*
pER371	2.7	*Streptococcus thermophillus*
pER341	2.8	*Streptococcus thermophillus*
pND103	3.5	*Streptococcus thermophillus*
pSt04	3.1	*Streptococcus thermophillus*
pER1-1	3.4	*Streptococcus thermophillus*
pJ34	3.4	*Streptococcus thermophillus*
pSt08	7.5	*Streptococcus thermophillus*
pt38	2.9	*Streptococcus thermophillus*
pMV158/ pE194		
pMV158	5.5	*Streptococcus aggalactiae*
pE194	3.7	*Staphylococcus aureus*
pSH71	2.1	*Lactococcus lactis*
pWV01	3.3	*Lactococcus lactis*
pCI411	2.9	*Leuconostoc lactis*
pC1305	8.7	*Lactococcus lactis*
pA1	2.8	*Lactobacillus plantarum*
pBM02	3.9	*Lactococcus lactis* subsp. *Cremoris*
pLF1311	2.4	*Latobacillus fermentum*
pLC2	2.6	*Latobacillus curvatus*
pLA106	2.9	*Latobacillus acidophilus*
pSMQ172	4.2	*Streptococcus thermophillus*
pSN2		
pSN2	1.3	*Staphylococcus aureus*
pT48	2.1	*Staphylococcus aureus*
pNE131	2.1	*Staphylococcus epidermidis*
pIM13	2.1	*Bacillus subtilis*
pE12	2.2	*Staphylococcus aureus*
pE5	2.1	*Staphylococcus aureus*
pTX14-3 (Andrup *et al.*, 2003)		
pTX14-3	7.6	*Bacillus thuringiensis*
pTX14-2	6.7	*Bacillus thuringiensis*
pG12	9.7	*Bacillus thuringiensis*

1.3.1.1. Rolling circle replication mechanism

Rolling circle replication is an asymmetric process because synthesis of the leading strand and synthesis of the lagging strand are not coupled. Replication through RCR mechanism is completed by SS-DNA formation. Therefore, the presence of SS-DNA intermediate indicates that plasmid replicates through RCR mechanism. The following points summarizes the RCR mechanism (Khan 2005)

1. Replication is initiated by plasmid encoded Rep protein, which interacts with its specific bind sequence and introduces a site specific *nick* at double-stranded origin (*dso*)
2. Tyrosine residue of Rep protein is attached to the 5′ phosphate. Rep protein also recruits a DNA helicase through a specific protein-protein interaction. The helicase then unwinds the DNA and SSB protein coats the displaced single stranded DNA.
3. Free 3′ OH end at the *nic*-site is used as a primer for leading strand synthesis by DNA polymerase III
4. Elongation from the 3′ OH end, accompanied by the displacement of the parental plus strand, continues until the replisome reaches the reconstituted *dso*, where a DNA strand transfer reaction takes place to terminate leading strand replication.
5. Following this, a series of cleavage and rejoining events generates a new closed double stranded circular DNA and a SS-DNA intermediate, which correspond to the parental plus strand
6. Finally SS-DNA is converted into DS-DNA by host proteins initiating at the *sso.*
7. The last step is the supercoilling of the replication products by host DNA gyrase.

1.3.1.1.1. Essential regions/domains and protein for rolling circle replication

1.3.1.1.1.1. Double-stranded origin (*dso*)

Double strand origin is a polynucleotide sequence (less than 100bp), which contains both binding and *nick*- site for Rep protein. The nick-site is highly conserved in the *dso* of the RCR plasmids belonging to a particular family, but not true for Rep-binding site in the *dso* (Khan 2005). The two regions (bind and *nick*-site) in the *dso* sequence of RCR plasmids can either be adjacent to each other, as exemplified by the plasmids of the pC194/pUB110 families, or be separated by a spacer region of 13 to 100bp (Khan 1995, del Solar 1998) as with pMV158 family. The *dso* of RCR plasmids contain direct and inverted repeats and are able to form hairpin structures. In the pT181 and pC194 families, the binding region is an inverted repeat IRIII adjacent

to the *nic*-site (loop region of hairpin structure IRII) (del Solar 1998). In the pMV158 family, the binding region is a set of two or three direct repeats separated from the *nic*-site (loop region of hairpin structure (IRI)) by the spacer sequence (del Solar 1998). RCR plasmids are grouped into five important families, based on their highly conserved *nic*-region similarity. These are pT181, pC194 and pMV158, pSN2, pTX14-3 (Shareck *et al.*, 2004; Khan, 1997). The plasmids belong to these families are listed in table 1.2.

1.3.1.1.1.2. Single-stranded origin (*sso*)

The single-stranded origins are noncoding specific sequence, which have a potential to generate extensive secondary structures. Sequence homologies and functional analyses have revealed the existence of four types of *ssos* in RCR plasmids (Khan, 2005).

1. *ssoA* present in several plasmids such as pT181, pC194, pLS1, pE194, and pIJ101
2. *ssoU* type origin was reported in plasmids pUB110
3. *ssoW* origin was found in lactococcal pWV01 plasmid
4. *ssoT* most commonly found in *Bacillus* plasmid such as pBAA1, and pTA1060

The *Streptococcus* pMV158 plasmid contains both *ssoA* and *ssoU*, but its derivative plasmid pLS1 contains *ssoA* only (Lorenz-Diaz and Espinosa, 2009). The *ssoA,* and *ssoW* type origins are functional only in their native hosts (Khan, 2005). *ssoT* and *ssoU* could support the lagging strand synthesis in several different hosts (Khan, 2005). The ssoU origin is very efficient in various Gram-positive bacteria such as *Bacillus subtilis, Streptococcus pneumoniae, Lactococcus lactis, Staphylococcus aureus* (Kramer *et al.,* 1999; Lorenz-Diaz and Espinosa, 2009). The *ssoU* origin is unique in the sense that it functions efficiently as signal for SS-DNA to DS-DNA conversion during plasmid rolling circle replication in a broad range of Gram-positive bacteria. Therefore, the presence of the *ssoU* sequence is an important factor in determining the promiscuity of RC plasmids (Kramer *et al.,* 1999; Lorenz-Diaz and Espinosa, 2009). Two conserved sequences have been reported in the *ssoA* type origins: 1. a 6-nt consensus sequence (5′- TAGCGA/T-3′) referred to as CS-6, located

within the terminal loop of the major secondary structure of *ssoA*. 2. a recombination site (RS_B), that is known to be involved in inter-plasmid recombination and play an important role in *ssoA* activity (del Solar *et al.,* 1998; Khan, 2005). The conversion of SS-DNA (released after leading strand replication) to DS-DNA requires *sso* and host proteins such as RNAP, DNA Pol I, DNA pol III, DNA gyrase etc (Lorenz-Diaz and Espinosa, 2009). The host protein (RNAP) binds to the SS-DNA containing RS_B sequence and synthesizes an approximately 20-nt RNA primer (pRNA) within the vicinity of RS_B. The conserved CS6 sequence serves as termination site of pRNA. The host DNA polymerase I then initiates DNA synthesis by using the RNA primer, and subsequent DNA synthesis by DNA polymerase III (Khan, 2005; Lorenz-Diaz and Espinosa, 2009). Finally DNA ends are joined by DNA ligase and resultant DS-DNA is converted to the supercoiled form by DNA gyrase (Khan, 2005).

1.3.1.1.1.3. Replication Initiator Protein

Replication proteins of RCR plasmids have DNA strand transferase enzymatic activity, so that they are able to cleave and to join plasmid DNA. There are many RC initiator proteins which have been identified in RCR plasmids of Gram-positive bacteria. These Includes RepA (in pC194), RepB (in pMV158), RepC (in pT181), RepD (in pC221) and RepH (in pUB110) (del Solar *et al.,* 1998; Khan, 2005).

All RCR rep proteins have mainly two domains, nicking-closing and DNA binding domains (Khan, 2005). Nicking domain (contains conserved motif3: UXX**Y**UXKXX) contains active tyrosine residue, which is involved in DNA nicking at *nic*-site of *dso* (Ilyina and Koonin, 1992; Khan, 2005), This domain is conserved in initiator proteins belonging to the same plasmid family. Binding domains of initiator proteins are not well conserved among members of same plasmid family. Therefore, Rep proteins are highly specific for the replication of their cognate plasmid (del Solar, 1998; Khan, 2005). RCR plasmids also contains conserved His motif (Motif2: HUH), involve in metal ion co-ordination (del Solar, 1998; Ilyina and Koonin, 1992).

1.3.2. Theta replicating plasmids

Theta-replicating plasmids are generally medium and large-size plasmids that encode important metabolic functions, enzymes such as lactase-protease (pUCL22), lactase (pSK11L), and citrate permease (pSL2), bacteriophage resistance (pCI528),

exopolysaccharide production (pNZ4000), and pediocin production (pMD136) (Shareck *et al.*, 2004). Theta mode of replication has also been reported in some cryptic plasmid such as pCI305, pWV02, and pVS40. Few of the theta replicating plasmids are small size including a 4.4 Kb p4028, 2.7 Kb pTXL1 and 1.9 Kb pMB1. Theta replicating plasmid do not produce SS-DNA intermediates, therefore these plasmids showed greater segregational and structural stability (Shareck *et al.*, 2004)

1.3.2.1. Theta mechanism

Replication through theta mechanism involves the melting of the DNA strands, synthesis of primer RNA (pRNA) and initiation of DNA synthesis by covalent extension of the pRNA. DNA synthesis can start from one to several origin of replications. In theta mechanism, replication can be either uni- or bi-directional. Replication via Θ (theta) shaped DNA structure intermediates confirms the theta mechanism (del Solar, 1998)

1.3.3. Conjugative and Mobilizable Plasmids

Conjugal transfer system is the transfer of genetic material from donor to recipient cell. Conjugative and mobilizable plasmids may be the main vehicles for this so called horizontal gene transfer or promiscuous transfer (Lorenzo-Diaz *et al.*, 2011). Because they can pick up genetic information from one host and transfer it to the unrelated micro-organisms. The mechanism for transferring the DNA is mediated by multi-protein complex (the relaxosome) (Garcillan-Barcia *et al.*, 2009). Initiation of transfer requires the cleavage of the transferring DNA at the specific palindromic sequence called *ori*T by a protein termed relaxase. As a result of reaction, the relaxase become covalently bound to the *ori*T DNA. This nucleo-protein complex is actively pumped into recipient cell by a plasmid encoded type IV coupling protein (T4CP) and the transferosome, a type IV secretion system (T4SS) (Garcillan-Barcia *et al.*, 2009; Lorenzo-Diaz *et al.*, 2011). **Conjugative plasmids** encode complete protein machinery (relaxase, T4CP, T4SS) required for conjugal transfer. Conjugative plasmid is also known as self transmissible plasmid (Garcillan-Barcia *et al.*, 2009; Lorenzo-Diaz *et al.*, 2011). They are the low copy number, small size plasmids. Enterococcal plasmids pAD1, pCF10, pAMβ1 are the representative of conjugative plasmids (Clewell and Francia, 2004; Francia *et al.*, 2004, Grohmann *et al.*, 2003).

Many small RCR plasmids only harbor an *ori*T, and the relaxase gene, these can only be transferred when they co-reside with the conjugative helper plasmid. These small size plasmids are known as **mobilizable plasmids** (Garcillan-Barcia *et al.,* 2009). Enterococal plasmid pAMα1 (9.75Kb) and streptococcal pMV158 and its homologues are representative of mobilizable plasmids (Clewell and Francia, 2004; Grohmann *et al.,* 2003; Weaver *et al.,* 2002).

1.3.3.1. Essential genetic element and protein responsible for conjugal transfer

1.3.3.1.1. Relaxase Protein:

Relaxases are the important element of the conjugative and mobilizable plasmids for the mobilization of SS-DNA from donor to recipient cell. Relaxase contains two or more protein domains. The N-terminal moiety of relaxases harbor endonuclease and DNA binding domains (Garcillan-Barcia *et al.,* 2009; Lorenzo-Diaz *et al.,* 2011). These domains usually contain two conserved motifs (Motif I & Motif III) (Garcillan-Barcia *et al.,* 2009). Motif I contains a catalytic Tyr residue, that reversibly attacks the DNA backbone in the relaxase catalyzed reaction (Garcillan-Barcia *et al.,* 2009; Lorenzo-Diaz *et al.,* 2011). Motif III (3H motif, HXDEXXPHUH; X=any residue, U =hydrophobic residue) contains three His residues involved in divalent (Mg2+, Mn2+, Cu+, Zn2+, Ni2+) metal ion co-ordination (Lorenzo-Diaz *et al.,* 2011). Metal bound to His residues are required for activating motif I Tyr residue for its nucleophilic attack at the *nic* site (Byrd and Matson, 1997; Lorenzo-Diaz *et al.,* 2011). The C-terminal moiety of relaxases contains a DNA-helicase or primase domain and/or domain for other functions such as membrane association and protein-protein interactions (Lorenzo-Diaz *et al.,* 2011). The structures of the N-terminal nuclease domains of the four conugative relaxases are known (Monzingo *et al.,* 2007). These relaxases are MobA (RII62 plasmid), TrwC (R388 plasmid), TraI (Fplasmid), TraI (pCU1plasmid) (Datta *et al.,* 2003; Guash *et al.,* 2003; Lorenzo-Diaz *et al.,* 2011; Nash *et al.,* 2010). The relaxases of conjugative and mobilizable plasmids from Gram-positive bacteria mainly belong to two families, the IncQ type family and the RCR pMV158-tpe family (Table 1.3) (Clewell and Francia, 2004).

Table 1.3: Plasmid grouping according to relaxase similarities (Clewell and Francia, 2004)

IncQ-like plasmid	pIP501	S. agalactiae	AAA99466.1
	pRE25	E. faecalis	CAC29179
	pSK41	S. aureus	AAC61938.1
	pMRC01	L. lactis	NP_047290.1
	pG01	S. aureus	AAB09712.1
pMV158-like plasmid	pMV158	S. agalactiae	AAA25387
	pVA380	S. ferns	AAA19677.1
	pSSU1	S. swis	BAA83679
	pUB110	S. aureus	AAF85649
	pIP823	L. monocytogenes	AAA93296
	pIP1714	S. cohnii	AAC61672
	pSMQ172	S. thermophillus	AAK83121
	pER13	S. thermophillus	NP115336.1
	pLB4	L. plantarum	AAA25252
	pLAB1000	L. hilgardii	A35390
	pBM02	L. lactis	AAK13009.1
	pS86	E. faecalis	CAA11139
	pBC16	B. cereus	AAA84921
	pSBK203	S. aureus	AAA79055.1
	pE194	S. aureus	QQSA4E
	pT181	S. aureus	NP040472.1
	pKH6	S. aureus	NP053796.1
	pLC88	L. casei	AAA74581.1
	pGI2	B. thuringiensis	P10025
	pTX14-1	B. thuringiensis	NP054010
	pTX14-2	B. thuringiensis	NP795748.1
	pTX14-3	B. thuringiensis	Q03980
	pTB19	Bacillus sp.	AAA98305.1
	pTB53	Bacillus sp.	BAA03580.1
	pTB913	Bacillus sp.	AAA98307
	pTA1015	B. subtilis	NP_053784
	pTA1060	B. subtilis	AAC44416
	pUH1	B. subtilis	A48371
	p1414	B. subtilis	NP049443.1

1.3.3.1.2. Origin of transfer:

An origin of transfer is a short sequence of the plasmid DNA that is necessary for transfer of bacterial plasmid from Donor cell to recipient during conjugation (Grohmann *et al.,* 2003). These short sequences contains two and more than two inverted repeats and are able to form potential secondary structure similar to *dso*. An

oriT consists of the three functional domains: a nicking domain, a transfer domain, and a termination domain. A multi-protein complex called the relaxosome assembles at/around *oriT*. A relaxase catalyzes cleavage of a specific phosphodiester bond at *nic* site of oriT, and becomes covalently linked through a tyrosyl residue to the 5' terminus of the cleaved strand (Clewell and Francia, 2004; Lorenzo-Diaz *et al.*, 2011). The reaction catalyzed by a relaxase is properly referred to as trans-esterification reaction (Byrd and Matson, 1997). Relaxase then moves in the 5' to 3' direction on the plasmid, unwinding the DNA in a helicase-like fashion, until it comes a full circle back to the *oriT* where the relaxase recognizes a termination domain that causes it to dissociate from the DNA. Termination of DNA is achieved by second trans-esterification reaction, in which the 3' OH of the nicked strand attacks the phosphotyrosyl linkage, resulting in resealing of the DNA strand and release of the protein in an unaltered form (Clewell and Francia, 2004).

Specific nick sites have been identified on the conjugative plasmids pIP501 (*Streptococcus*), pG01 (*Staphylococcus*), pRE25 (*Enterococcus*), as well as on the mobilizable plasmid pMV158 (*Streptococcus*) (Clewell and Francia, 2004; Grohmann *et al.*, 2003). The sequence of the *nic* regions of the conjugative Gram-positive bacterial plasmids (pIP501, pG01 & pRE25) were shown to be similar to the *nic* sequence of the IncQ type plasmids (RSF1010, R1162) of Gram-negative bacteria (Grohmann *et al.*, 2003). The pMV158 plasmid is the representative of an *oriT* family, mainly made up of Gram-positive RCR mobilizable plasmids (Grohmann *et al.*, 2003).

1.3.3.2. RCR Mobilizable plasmids: the pMV158 family

RCR Mobilizable plasmid pMV158 has broad host range capacity. This plasmid is functional in *E. coli* as well as other Gram-positive bacteria. The plasmid pMV158 encodes a MobM protein (DNA relaxase), which binds to the specific *oriT* and perform nicking-joining reactions during DNA transfer. The MobM is the first DNA relaxase from Gram-positive bacteria that has been purified and characterized (Guzman and Espinosa, 1997; Lorenzo-Diaz *et al.*, 2011). There are nearly 50 Mob proteins that show a high degree of similarity to MobM. Some of them are the Mob proteins from the staphylococcal plasmids pUB110, pE194, pT181, and pC221

(Grohmann *et al.*, 2003). The MobM proteins belong to the MOBv relaxase family, which encompasses nearly 100 members (Garcillan-Barcia *et al.*, 2009). The MOBv relaxase family is divided into five clades like MOBv1, MOBv2, MOBv3, MOBv4, & MOBv5. The clade MOBv1 is the most populated and it comprises the relaxases encoded by mainly RCR plasmids (Garcillan-Barcia *et al.*, 2009). The prototype is the streptococcal plasmid pMV158 (Garcillan-Barcia *et al.*, 2009). MobM protein contains N-terminal binding and nicking domain and C-terminal protein-protein interactions domain (Lorenzo-Diaz *et al.*, 2011). Binding and nicking domain contains three conserved motifs motif I (HxxR; x = any residue), motif II (NYD/EL), and motif III (H-x-DE-xx-PH-X-H; x = any residue) (Lorenzo-Diaz *et al.*, 2011). The function of motif I is unknown, motif II contains catalytic tyr residue involved in nicking at the *nic* site of *ori*T, motif III, also known as 3H motif, probably involved in divalent metal ion co-ordination (Lorenzo-Diaz *et al.*, 2011). The two motifs; motif I (HxxR; x = any residue) and motif III (H-x-DE-xx-PH-X-H; x = any residue) are well conserved in MOBv1 relaxases (Garcillan-Barcia *et al.*, 2009). Guzman and Espinosa (1997) have revealed the presence of two inverted repeats in the *ori*T of pMV158 plasmid. They have experimentally mapped the *nic*-site at *ori*T, and found that the loop of the IR 2 contains the *nic* site. Recently Lorenzo-Diaz *et. al.* (2011) has reported the presence of three inverted repeats in the *ori*T of pMV158 (Fig 1.1). The third inverted repeat (IR 3) includes IR 1 plus 5 and 8 bases up- and downstream respectively. They have found the binding site for N-terminal region of MobM protein (MobMN199) are IR 3 and IR1 + 8 regions of *ori*T.

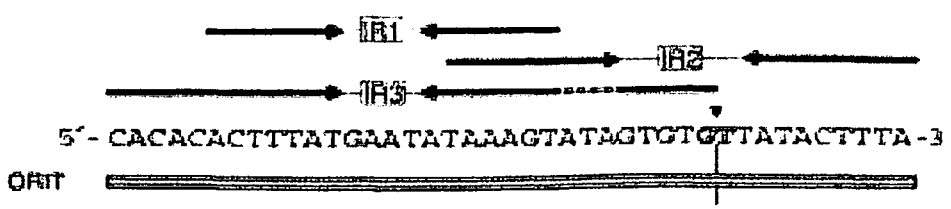


Fig. 1.1: The *ori*T (pMV158) sequence, indicating the three inverted repeats IR1, IR2, and IR3 (Lorenzo-Diaz *et. al.*, 2011)

1.4. Enterococcal plasmids

A large number of enterococcal plasmids ranging from small size plasmids to megaplasmids composed of different functional modules, responsible for plasmid replication, maintenance, conjugal transfer and phenotypic traits such as resistant to antimicrobials (Wardal *et al.*, 2010).

Three plasmid types are known to replicate in enterococci (Wardal *et al.*, 2010):

1.4.1. Broad host range RCR plasmids

To our knowledge, there are few functional RCR plasmids native to enterococci. The pAMα1 is a naturally occurring small size (9.75 Kb), multicopy, tetracycline resistant RCR plasmid identified in *E. faecalis* (Francia and Clewell, 2002). The growth of *E. faecalis* containing pAMα1 resulting the amplification of a plasmid containing tetracycline (tet) resistant gene (tandem repeats of 4.1 Kb segments of DNA containing tet determinant), while growth in the absence of tet resulted in deletion of the gene. The pAMα1 consisting of pAMα1Δ1 and pAMα1Δ2 joined by recombination sequences (RS I and RS II) (Francia and Clewell, 2002; Weaver *et al.*, 2002). A recombination event between these sequences initiates the amplification. The pAMα1Δ1 contains tet resistant determinant (tetL), repB, and mobB genes (Francia and Clewell, 2002). The pAMα1Δ1 was found to closely resemble with pBC16 and other plasmids of *Bacillus* and pUB110 of *S. aureus* (Clewell and Francia, 2004; Francia and Clewell, 2002). The pAMα1Δ2 (deleted pAMα1Δ1 portion from pAMα1 plasmid) contains mobE and repE genes (Francia and Clewell, 2002). The pAMα1Δ2 shows a high degree of similarity with the small cryptic plasmid pS86 of *E. faecalis* (Clewell and Francia, 2004; Francia and Clewell, 2002). The Mob genes of pAMα1Δ1 and pAMα1Δ2 are closely related with the mobM of pMV158 plasmid. Garcia-Miguara *et al.* (2009) has reported the presence of small size mobilizable plasmid pRI1 in *Enterococcus faecium.* The pRI1 is a 6.038 Kb plasmid contains putative rep and mob genes. The plasmid pRI1 replicates via RCR mechanism.

1.4.2. Inc18 plasmids

Inc18 plasmids are a family of broad host range low copy number conjugative plasmids that occur naturally in *Enterococcus* and *Streptococcus* spp. Three plasmids pIP501 from *Streptococcus agalactiae*, pSM19035 from *Streptococcus pyrogens* and pAMβ1 and pRE25 from *Enterococcus faecalis* are the representative of a family of Inc18 group plasmids (Clewell and Francia, 2004; Kurenbach *et al.*, 2003). The size of these plasmids is generally between 25 and 50 Kb (Clewell and Francia, 2004; Weaver *et al.*, 2002). These plasmids carry macrolides, lincosamides, and the streptogramin B (MLS) resistance genes (Zhu *et al.*, 2010). These resistant genes can be transferred to broad range of bacteria, including streptococci, lactococci, staphylococci, and enterococci (Zhu *et al.*, 2010). There are the reports of the vancomycin resistant enterococci (VRE) isolates which carry vanA plasmids such as pIP819 and pWZ909 (Zhu *et al.*, 2010). These vanA plasmids showed the similarity with a family of Inc18 group plasmids.

1.4.3. Pheromone responsive plasmids (limited to enterococci)

Pheromone responsive plasmids are narrow host range, self conjugating plasmids, whose conjugal transfer are induced by pheromones (extra cellular small peptides) secreted by recipient (plasmid free) cells (Clewell and Francia, 2004). In response to pheromones, donor cell produces proteinaceous structures on the cell surface called aggregation substance (AS) which bind to enterococcal binding substance (EBS) present on the surface of recipient (Wardal *et al.*, 2010). During this process, a mating channel between donor and the recipient is formed, which enables the transfer of plasmid DNA. The pheromone responsive plasmids are in the range from 37 Kb to 128 Kb and commonly found in *Enterococcus faecalis* (Wardal *et al.*, 2010). The plasmids pAD1, pCF10, pPD1 and pAM373 are the most extensively studied pheromone responding plasmids (Wardal *et al.*, 2010). The plasmid pAD1 was the first discovered pheromone responsive conjugative plasmid (Clewell, 2007).

1.5. Vector derived from lactic acid bacteria plasmids

Studies based on the characterization of natural plasmids from lactic acid bacteria have made it possible to develop various cloning and expression vectors.

Currently used vectors for lactic acid bacteria and other Gram-positive bacteria are derived from the following replicons

1. lactococcal RCR plasmids of pSH71 and pWV01 (Miyoshi *et al.*, 2010; Shareck *et al.*, 2004): vectors derived from the replicons of pSH71 and pWV01 suffer from segregational instability due to their RCR mechanism, and can rarely maintain the large size DNA fragments. pSH71 and pWV01 derived vectors are suitable for the cloning and expression of small DNA fragments. Because of their small size , propensity for carrying antibiotic resistance genes and relatively broad host range, these vectors have provided useful starting material for the construction of variety of cloning and expression vectors.
2. Lactobacilli RCR plasmids of pA1, pLC2, pLF1311 and pGT633 (Shareck *et al.*, 2004): vectors derived from these replicons could be functional in *Lactobacillus*, other Gram-positive bacteria and *E. coli*.
3. Streptococcal pIP501 and enterococcal pAMβ1 and their derivative (Miyoshi *et al.*, 2010; Shareck *et al.*, 2004): The pIP501 and pAMβ1 are theta replicating plasmids and their derivatives exhibits segregational stability. They are conjugative plasmids and resistant to macrolides, lincosamides and spectogramin B and antibiotics. Vector derived from these plasmids and their derivatives can clone the large fragment of DNA.
4. In addition of these replicons, cryptic plasmids originating from other lactic acid bacteria have been used for construction of several narrow and broad host range vectors.

1.5.1. Enterococcal cloning and expression vectors:

Enterococcal cloning and expression vectors are basically derived from the replicons pVA380-1(streptococcal), pIP501 (streptococcal) (Kurenbach *et al.*, 2003), pAMβ1 (enterococcal) (Kurenbach *et al.*, 2003), and pAMα1 (enterococcal) (Weaver *et al.*, 2002). Some of the enterococcal vectors are developed as improved shuttle cloning and regulated expression vectors (Weaver *et al.*, 2002). These vectors are able to replicate in enterococcus, other Gram-positive bacteria and in *E. coli*. pDL276, pDL278, pVA838 are shuttle cloning vectors, they replicates through RCR mechanisms and confers resistance to various antibiotics. They are constructed from

the streptococcal replicon pVA380-1 and *E.coli* replicons pUC19 (for pDL276, pDL278) and pACYC184 (for pVA838) (Weaver *et al.*, 2002). The pAM401 and pWM401 is a widely used shuttle vector that can be maintained stably both in *E. coli* and in *E. faecalis* (Fujimoto and Ike, 2001). These vectors are constructed by streptococcal pIP501 and *E. coli* pACYC184 replicons (Weaver *et al.*, 2002). A shuttle vector pHY300PLK contains tet and amp resistance genes, are derived from the replicons of pAMα1 (enterococcal RCR mobilizable plasmid) and pACYC177 (*E. coli*) (Weaver *et al.*, 2002). This vector is able to replicate in *Enterococcus, Bacillus* as well as in *E. coli*. The pTRKH3 a 7.3 Kb size plasmid shuttle vector derived from *Escherichia coli* plasmid p15A origin and *Enterococcus faecalis* plasmid pAMβ1 origin, incl. repD and repE (Papagianni *et al.*, 2007).

Apart from the shuttle cloning vectors, enterococcal expression and promoter probe vectors have also been reported. Some of the expression vectors are pMSP3535 (replicons: *E. coli* ColE1 and enterococcal pAMβ1), pMGS100, and pMGS101 (*E. coli* pACYC184 and streptococcal pIP501) (Weaver *et al.*, 2002). A low copy number vector designated pTCV-lac is used to analyze regulatory elements in Gram-positive bacteria (Poyart and Trieu-Cuot, 1997). The main components of this vector are (a) the origin of replication of pACYC184 and of the broad host range enterococcal plasmid pAMβ1 (b) the transfer origin of IncP plasmid RK2 (C) selection marker erythromycin and kanamycin (d) promoterless β-galactosidase encoding gene with Gram-positive RBS (Poyart and Trieu-Cuot, 1997).

1.6. Controlled expression systems

Continuous high level of protein production in the cell leads to intracellular accumulation, aggregation or degradation of protein in the cytoplasm, which is deleterious to the cells (Zhou *et al.*, 2006). To circumvent these problems, gene expression systems require the regulated promoter and/or promoter signal sequence units functioning in a host. Regulated promoters control the gene expression in the host by an inductor, a repressor, and environmental factors, such as temperature, pH, and salt concentrations (Shareck *et al.*, 2004). Gene transcription starts when the sigma (σ) subunit of RNA polymerase recognizes the promoter region. The promoter regions of Gram-positive bacteria consists with two primary elements of -35 and -10 regions and TG-motif of -16 region and the AT rich UP element (Miyoshi *et al.*,

2010; Voskuil and Chambliss, 1998). The principle σ factor of lactic acid bacteria in particular *L. lactis*, σ^{39} recognizes the consensus sequences TTGACA (-35) and TATAAT (-10). Similar consensus sequences are recognized by *E. coli* σ^{70} and *Bacillus subtilis* σ^{A} (Jeong *et al.*, 2006; Miyoshi *et al.*, 2010). The TG motif of -16 region is positioned 1 base upstream to -10 region has been reported in number of Gram-positive bacterial promoters (Voskuil and Chambliss, 1998). This motif is also known as extended -10 promoters in *E. coli*. The extended -10 promoters lack an identifiable -35 region but are transcribed by RNAP $E\sigma^{70}$ (Voskuil and Chambliss, 1998).

The UP element, a component of bacterial promoters located upstream of the -35 hexamer (from positions -59 region to -38 region). The consensus sequence for UP element is -59 nnAAA(A/T)(A/T)T (A/T)TTTT nn AAAAnnn-38 (Estrem et al., 1998). The presence of UP element sequences and the -16 region may functions as a co-operative or additive manner. Therefore, promoters containing the -16 region, primary elements -35 and -10 regions, UP elements and other conserved sequences comprise a class of strong promoters (Estrem et al., 1998; Voskuil and Chambliss, 1998). Gene expression can be controlled at translational level.

Initiation of translation requires a ribosome binding site (RBS). The efficiency of translation or protein biosynthesis is affected by the primary and secondary structures of mRNAs in the region of the binding site of the 30S ribosome subunit. Shine-Dalgarnov (S/D) sequence together with initiation codons (AUG or GUG) constitutes the ribosome binding site. The primary structure of typical RBS is $AGGN_{6-9}ATG$. The distance between S/D sequence and the AUG initiation codon is important for determining the translational efficiency. As the distance between S/D sequence and the AUG initiation codon increases, translational efficiency decreases. The secondary structure of the translational initiation regions (TIR) also play important role in the protein biosynthesis. This fact can be explained by an example in which, the expression levels of the t-antigen has been determined under control with the same promoter, in vector pTR436 and HP1. The expression level of t-antigen is found many fold high in HP1, even the distance between S/D sequence and the start codon (ATG) in the pTR436 and HP1 were same. They were differing only in two nucleotides in the translational initiation regions (TIR). Freier et al. (1986) have shown that efficient

translation requires an easily accessible AUG start codon residing in a single stranded rather than a double stranded region of the hairpin loop of the translational initiation region (TIR). The substitution of AA for CC in plasmid HP1, for example exposes the start codon by bringing it into an easily accessible single-stranded region, and hence expression is higher in HP1 than pTR436. Several regulated expression systems in lactic acid bacteria are described, they are based on promoters controlled by sugars (*lac* promoter), salt (*gadC* promoter), temperature upshift (*tec* phage promoter), acid inducible (P1, P3 & P170 promoters), phage infection (Φ31 promoter) and most widely used *nisA* promoter (Shareck *et al.*, 2004). Details of some of the systems are given below.

1.6.1. Sugar inducible expression system

Most sugar inducible expression system are based on the use of the *lac* promoter of the lactose operon. This promoter is controlled by auto-regulated LacR repressor (Marelli and Magni, 2010). The *lac* promoter is induced in the presence of lactose. Induction by lactose is effected by the intermediate tagatose-6-phosphate that inactivates the LacR repressor (Marelli and Magni, 2010). However this system is hampered by a low level of induction. To, overcome this problem lactose inducible *lac/T7* promoter based expression system has been developed (Miyoshi *et al.*, 2010). This system composed of three vectors, which combined the elements of lac operon with elements of bacteriophage T7 of *E. coli* (Miyoshi *et al.*, 2010). In one vector T7 RNA pol ORF is under control of the *lac* promoter. When lactose is added in the medium, T7 RNA pol synthesis is induced, which activates the expression of gene of interest controlled by T7 promoter in the second vector. However a third vector (lac operon is necessary) is required to metabolize lactose in the medium (Miyoshi *et al.*, 2010).

1.6.2. Expression based on phage promoters:

The repressor-operator system of r1t phage of *L. lactis* and genetic elements of phage Φ31 are the expression systems based on phage promoters.

In the first system, an ORF is placed under control of the phage promoter P_{orf5}. The promoter is repressed by phage protein Rro. The repressor protein Rro is cleaved in the presence of mutagen mitomycin C. Hence, mitomycin C releases Rro protein from

the P_{orf5} promoter and induces the expression of ORF (Miyoshi *et al.*, 2010; Nauta *et al.*, 1996).

In the second system, genetic elements of phage Φ31 such as origin of replication (*ori31)* and the promoter P_{15A10} has been used to develop plasmid based expression vector in *L.lactis* (Miyoshi *et al.*, 2010). ORF to be activated is cloned under the control of P_{15A10}, with *ori31* in the same vector. When phage Φ31 infects the *L. lactis*, number of copies of the vector is multiplied (multiplied due to presence of phage *ori31*) in the infected cell and hence level of expression of ORF by promoter P_{15A10} is also increased (Miyoshi *et al.*, 2010). The greatest disadvantage of this system is the need for cell infection to obtain induction

1.6.3. Acid inducible expression systems:

Three *Lactococcus lactis* promoters, P170, P1 and P3 are acid inducible promoters (Madsen *et al.*, 2005). The promoter P170 and P1 are strongly acid inducible promoters, they are consisted with -10 regions, extended-10 regions and three tetranucleotide boxes A, C and D (ACiD box) (Madsen *et al.*, 2005). The lack of -35 sequence in both promoters could explain their low basal activity at neutral pH. It was reported that trans-acting protein, RcfB, which bind to acid box and enhance transcription (Madsen *et al.*, 2005). The acid signal is required for activation of RcfB.

Promoter P3 is stronger than P1 and weakly acid inducible. This promoter contains partial -35 sequence, and consensus -10 region. In addition promoter P3 possesses boxes C and D. Due to absence of box A and presence of partial -35 sequence this promoter is weak acid inducible (Madsen *et al.*, 2005).

1.6.4. Nisin controlled expression system

Primarily nisin controlled expression systems (NICE) was developed in *L.lactis.* Later this system was developed for number of Gram-positive bacteria such as *Lactobacillus, Streptococcus, Enterococcus, Bacillu, Leuconostoc* etc. Nisin is an antimicrobial peptide, and its biosynthesis is controlled by a nisin gene cluster (*nisABTCIPRKFEG*) (Zhou *et al.*, 2006). The gene cluster contains three promoters, *nisA, nisF* and *nisR*. Among these promoter *nisA* and *nisF* are involved in nisin mediated autoregulation. During auto regulation process, nisin induces the

transcription of nisin gene clusters (Zhou *et al.*, 2006). This nisin mediated induction is based on the signal transduction by a two-component regulatory system, consisting of the sensor histidine kinase, NisK, and the response regulator NisR. When nisin binds to the NisK, NisK autophosphorylates and this activated NisK activates the response regulator NisR through phosphorylation. Activated NisR acts in the *nisA/nisF* promoter and induces gene expression (Kim and Mills, 2007). This auto-regulatory NICE system clearly indicates that the plasmid contains *nisRK* gene along with the *nisA* promoter can induce the expression of heterologous protein in the host (under control with *nisA* promoter). Several modifications of the NICE system have increased its utility. One of the most important improvements was the cloning of *nisRK* gene and the *nisA* promoter in the LAB-*E. coli* shuttle vector (pMSP3535) (Kim and Mills, 2007). This vector has pAMβ1 enterococcal replicon, which is stably maintained in the various LAB species.

Aims

To the best of our knowledge, limited reports suggesting that, promiscuous plasmids exist in the Gram +ve LAB which can also propagate in Gram –ve organisms. Therefore, we were interested in identifying promoters which would be efficient in both Gram +ve and Gram –ve organisms so as to enable creation of novel vectors with multiple use.

Objectives

1. Identification and screening of LAB from different sources for the presence of plasmids
2. Characterization of a small size plasmid screened from natural isolates of lactic acid bacteria for ORFs, functional secondary structures and replication mechanism
3. Identification and screening of promoter regions from the LAB plasmid
4. Analysis of the strength of these promoter sequences in *E.coli*

Organization

The thesis is organized according to aims as mentioned above. Chapter 1 gives the introduction to the topic based upon the literature available till date and gap in existing research; Chapter 2 explains about the methodology, followed for achieving the above mentioned objective; Chapter 3 deals the identification and screening of LAB from different sources for the presence of plasmids; Chapter 4 communicates the structural and functional organization of one of the plasmid designated pNJAKD, isolated from *E. faecium* DJ1; Chapter 5 details the characterization of promoter fragments identified in pNJAKD plasmid; Chapter 6 lists the conclusion drawn out of the complete work.

Chapter 2

Materials and Methods

2. MATERIALS AND METHODS

2.1. GENERAL PROTOCOL

2.1.1. Plasmid DNA isolation from *E. coli* [DH5α, JM101, BL21(DE3)]

Plasmid DNA from *E.coli* was extracted by alkaline lysis method, when needed plasmid was further purified by using Qiagen plasmid mini kit and Qiagen-tip-100

2.1.1.1. Alkaline lysis method

Reagents used:

1. Solution I: 50mM Glucose, 10mM EDTA (pH 8.0), 25mM Tris-Cl (pH 8.0)
2. Solution II: 0.2N NaOH, 1 % SDS
3. Solution III: 3M Sodium Acetate, Glacial Acetic acid. Adjust pH to 5.2
4. Solution IV: Tris -saturated Phenol (pH 8.0), Chloroform:Isoamylalcohol (24:1)
5. Absolute alcohol (99.5%) and 70% Ethanol
6. RNase A (Sigma, 10 mg/ml)
7. 1 X TE buffer (pH 8.0)
8. Luria-Bertani broth (LB broth, HIMEDIA)
9. Antibiotic (Ampicillin sodium salt, HIMEDIA)

10 ml LB-AMP (100 μg ml^{-1}) media was inoculated with single colony of *E. coli* transformed with recombinant molecule and incubated at 37 °C for 14 h with shaking (200 rpm). 1.5 ml of 14 h old culture was taken in microfuge tube and centrifuge for 5 min at 5000 rpm. Supernatant was discarded and cell pellet was re-suspended in 0.1 ml GET buffer or solution I. To this, 0.2 ml freshly prepared lysis buffer or solution II was added, mixed gently (6-10 times) and incubated at room temperature for 5 min. To the above mix, 150 μl chilled solution III was added, mixed gently and incubated

on ice for 15 min. The lysed material was then spun at 10,000 rpm for 10 min at 4 °C and the supernatant was transferred to a fresh microfuge tube. A phenol: chloroform: isoamyl alcohol (25: 24: 1) treatment was then given to remove all the proteins. DNA was then precipitated overnight by adding twice the volume of 100% ethanol in the presence of 1/10 volume of 3M Na-acetate. After overnight precipitation, solution was centrifuged at 8000 rpm for 30 min at 4 °C. Supernatant was discarded and pellet was washed with 1 ml chilled 70 % ethanol, air dried and suspended in adequate amount of 1X TE buffer

2.1.1.2. Qiagen TIP -100 (Starter culture: 100 ml)

Reagents used:

1. Buffer P1 (resuspension buffer): 50mM Tris-Cl (pH 8.0), 10mM EDTA, 100µg ml^{-1} RNase A
2. Buffer P2 (lysis buffer): 200mM NaOH, 1% SDS (w/v)
3. Buffer P3 (neutralization buffer): 3.0M potassium acetate (pH 5.5)
4. Buffer QBT (equiulibration buffer): 750mM NaCl, 50mM MOPS (pH 7.0), 15% isopropanol (v/v), 0.15% triton® X-100 (v/v)
5. Buffer QC (wash buffer): 1.0M NaCl, 50mM MOPS (pH 7.0), 15% isopropanol (v/v)
6. Buffer QF (elution buffer): 1.5M NaCl, 50mM Tris-Cl (pH 8.5), 15% isopropanol (v/v)

100 ml overnight culture was taken in Oakridge tube and centrifuged at 6000 rpm for 5 min at 4 °C. Bacterial pellet was resuspended in 4 ml of buffer P1. Resuspended pellet was gently mixed with 4 ml buffer P2 and incubated at room temperature for 5 min. To, this 4 ml of chilled buffer P3 was added, mixed gently and centrifuged at 12000 rpm for 30 min at 4 °C. Supernatant was removed and centrifuged again at 12000 rpm for 15 min at 4 °C. Now again supernatant was removed and collected in Oakridge tube. This supernatant was used for loading Qiagen Tip 100. Before loading, Quiagen tip 100 was equilibrated with 4 ml of QBT buffer and then allowed the above collected supernatant to enter the resin by gravity flow. For washing, 2x10 ml buffer QC was applied to the sample loaded Quiagen tip. Elution of the plasmid

DNA was achieved by adding 5 ml buffer QF. Eluted plasmid DNA sample was collected in the Oakridge tube and precipitated by adding 3.5 ml (0.7 volume) isopropanol at RT. Sample was mixed gently and centrifuge immediately at 12000 rpm for 30 min at 4 °C. Supernatant was decanted and pellet was washed with 2 ml of 70 % ethanol at RT. The pellet was air dried and suspended in adequate amount of 1X TE.

2.1.2. Preparation of Competent Cells

Reagents used:

1. 0.1M $CaCl_2$ (Calcium Chloride solution)
2. 60 % Glycerol
3. Luria – Bertani broth (LB broth)

The bacterial cells were grown overnight at 37 °C with shaking and inoculated into 150 ml fresh LB broth in ratio of 1:20. This culture was incubated at 37 °C with shaking until the optical density (OD) at 600 nm reaches 0.4 to 0.5. This takes about 3 hours. The cells were pelleted out by centrifugation at 6000 rpm for 10 min at 4°C in a pre-cooled rotor. It is important that from this point onwards the cells are not allowed to warm up. The pellet was re-suspended in 37.5 ml (1/4 volume) ice - cold 0.1 M $CaCl_2$ (by gently pipetting in and out repeatedly) and allowed to stand on ice for 20 minutes with occasional shaking. The suspension of cells were spun at 6,000 rpm for 10 minutes at 4 °C and re-suspended in 2 ml of ice - cold 0.1 M $CaCl_2$. To this, Glycerol was added, mixed and the cells were aliquoted and stored at -70°C till use (Sambrook *et al.*, 1989).

2.1.3. Transformation of *E. coli* with Plasmid/ Ligation Product

10 µL (Approximately 50 - 100 ng) of the plasmid DNA was added to the 1.5 ml micro centrifuge tube containing 150 µl competent cells and incubated on ice for 45 minutes. The micro centrifuge tubes were rapidly transferred to a 42 °C water bath for a heat shock treatment for 2 min. These tubes were replaced on ice. 1 ml LB medium, pre-warmed to 37 °C was added to each tube of cells immediately after the heat-shock. These tubes were incubated at 37 °C for 1 h. After incubation, the transformed

cells were pelleted out by centrifugation at 3000 rpm for 5 min. The amount of LB added was discarded and pellet was suspended in the remaining LB media. Content of each tube were spreaded on to LB agar plates with appropriate antibiotics and incubated over night at 37 °C (Sambrook *et al.*, 1989).

2.2. SCREENING AND IDENTIFICATION OF LAB

2.2.1. Samples and Bacterial isolates

Samples (milk, water, soil, plant) were collected from different regions (Pilani, Nagpur, Delhi, Moradabad, Bikaner, Jabalpur, Jodhpur) of India during March, 2005 in sterile 15 ml centrifuge tube (Tarson) and were brought to the laboratory at Center for Biotechnology, Department of Biological Science, BITS, Pilani. Plant and soil samples were blended with sterile Milli-Q water. Each sample was immediately transferred in to the sterile SSMD (yeast extract 0.5 g, peptone 0.5 g, sodium acetate 1 g, dextrose 1 g, skim milk 10 g make up to 100 ml with sterile Milli-Q water, pH: 6.8) media (Terzaghi and Sandine, 1975), and incubated at 30 °C for 24 h without agitation. After 24 h incubation, curd formation was observed in SSMD media (Fig. 2.1).

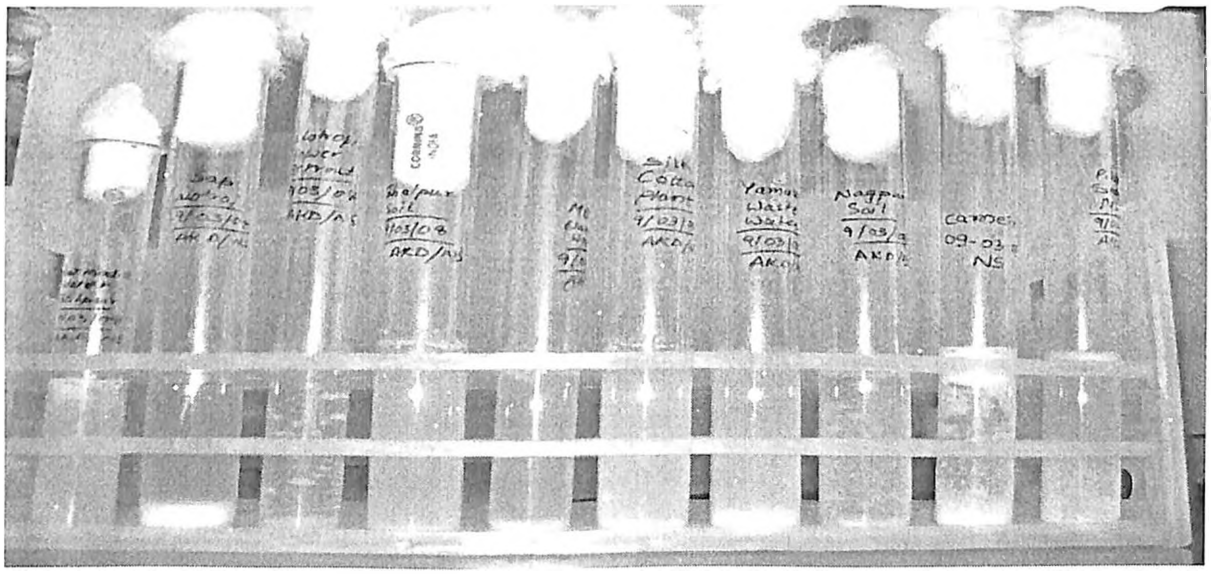


Fig. 2.1: Curd formation in SSMD media: Plant, soil, milk and water samples were inoculated in SSMD media, after 24 hours incubation curd was seen in test tubes containing media and samples

An uninoculated SSMD medium was used as a negative control. A loopful of curd from each sample was streaked on MRS- bromocresol purple (BCP, 0.005% final concentration)-agar plates for isolation of lactic acid bacteria (deMan *et al.,* 1960), and plates were incubated at 30 °C for 24 h. After incubation, single and isolated colonies were randomly picked and sub cultured twice on MRS-Agar media to obtain pure culture of the isolates. The pure cultures were grown on MRS agar at 30 °C for 24h, and transferred with 20% glycerol in MRS broth and stored as stock cultures at -80 °C for further analysis.

2.2.2. Morphological, physiological, and biochemical tests.

Reagents used:

1. Gram staining kit (HI-MEDIA)
2. 3% H_2O_2
3. 4.0 and 6.5% NaCl
4. p-aminodimethylaniline oxalate
5. Carbohydrates (Glucose, Fructose, Sucrose, Cellobiose, Maltose, Arabinose, Lactose)
6. MRS (HI-MEDIA) and M17 media
7. Antibiotics (HI-MEDIA): Ampicillin sodim salt, Tetracycline hydrochloride, Chloramphenicol, Vancomycin, Kanamycin sulphate, Streptomycin, Gentamycin,

2.2.2.1. Gram staining test: Morphology Identification and Test for Gram-positive bacteria

Gram staining procedure was done as follows:

1. A smear of 24 hour old bacteria culture was prepared and heat fixed
2. Flooded the slide with **Crystal Violet** (the *primary stain*).
3. After 1 minute, rinsed the slide with water.
4. Flooded the slide with **Iodine** (Iodine is a *mordant* that binds with Crystal violet and is then unable to exit the Gram+ peptidoglycan cell wall.)
5. After 1 minute, rinsed the slide with water.

6. Flooded the slide with **Acetone Alcohol**. (Alcohol is a *decolorizer* that will remove the stain from the Gram-negative cells.)
7. After 10 or 15 seconds, rinsed the slide with water. (Do not leave the decolorizer on too long or it may remove stain from the Gram-positive cells as well.)
8. Flooded slide with **Safrinin** (the *counterstain*).
9. After 1 minute, rinsed the slide with water.
10. Gently blot the slide dry. Viewed in microscope under oil immersion (100X)

After this staining procedure, the Gram + cells appear purple, having retained the primary stain.

2.2.2.2. Catalase Test: for catalase activity (a few drops of 3% H_2O_2 was added to a microbial broth culture)

2.2.2.3. Oxidase test: for cytochrome oxidase activity

1. Prepared 1% aqueous p-aminodimethylaniline oxalate solution
2. A strip of filter paper was soaked with 20 µl of 1% p-aminodimethylaniline oxalate solution
3. Bacterial colony was picked from agar plate and rubed onto moistened strip. A colour change (dark blue) within 10 seconds indicates a positive reaction.

2.2.2.4. Hetero/Homo fermentative Test:

1. Culture was Grown in M17 medium with 1% Glucose as a Carbohydrate
2. Dipped a red heated inoculating loop into the culture, the evolution of gas indicated that soluble CO_2 in the medium. Production of CO_2 gas from glucose (1% glucose) demonstrates the heterofermentative metabolism

2.2.2.5. Carbohydrate fermentation test (for Glucose, Fructose, Sucrose, Cellobiose, Maltose, Arabinose, Lactose):

Bacterial colony was inoculated in to M17 broth containing test sugar and incubated at 37 °C for 24 hours. A bright yellow color indicated the production of acid product from fermentation of sugar

2.2.2.6. Temperature tolerance:

Bacterial colony was inoculated in 10 ml MRS-broth and Growth was performed at 15, 40 and 45 °C.

2.2.2.7. NaCl tolerance

Bacterial colony was inoculated in M17 broth containing 4.5% & 6.5% NaCl. Growth was performed at 30 °C after 24 hours incubation.

2.2.2.8. pH tolerance

Growth was performed at pH 4.3, 6.2 and 9.6 in M17 or MRS broth at 30 °C for 24 hours

2.2.3. Antibiotic Test

Plasmid harboring strains were checked for antibiotic resistance. Test was performed by agar dilution method (ESCMID, 2000). Bacterial culture was streaked on MRS-BCP plate containing varying concenteration of antibiotic to be tested.

2.2.4. Antibacterial activity

Six LAB isolates; *E.faecium* DJ1, *E.faecium* DJ2, *E.faecium* DJ3, *Pediococcus pentosaceous*, *Staphylococcus epidermiditis* and *Weissella confusa* were examined for their antimicrobial activity against pathogenic microbes (*Bacillus cereus*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas putida*, *Escherichia coli*, *Salmonella* sp.) by bore well diffusion method (John *et al.*, 1999). Nutrient agar plates were made for pathogenic microbes. LAB isolate was grown on MRS-broth at 30 °C for 24 hours. 50 µl of cell free supernatant of a 24 hours old LAB culture was pipetted into the wells of pathogenic microbe agar plate. Zone of inhibition was measured in all pathogenic microbes plate for above mentioned LAB isolates.

2.3. SPECIES IDENTIFICATION OF LACTIC ACID BACTERIA

Genomic DNA isolation and then 16S-rDNA amplification and sequencing were performed for species identification of LAB

2.3.1. Genomic DNA isolation

Reagents used:

1. MRS broth (HI-MEDIA)
2. TE buffer: 10mM Tris-HCl, 1mM EDTA pH 8.0
3. 1% (w/v) SDS (HI-MEDIA)
4. Lysis solution: 1.0M Tris-HCl (pH 8), 0.5M EDTA, 5.0M NaCl, 1.0% (v/v) β-mercaptoethanol, 10% (w/v) SDS and 10 mg ml^{-1} Proteinase K (Sigma)
5. Tris saturated phenol (Genei & Sigma)
6. Chloroform (Merck)
7. Isoamyl alcohol (Merck)
8. 5 M ammonium acetate solution
9. Absolute alcohol (99.5%) and 70% ethyl alcohol
10. RNase A (Sigma, 10 mg/ml)

A single colony from each isolate was inoculated into 10 ml of the MRS-broth (kept in a 100 ml conical flask) and incubated for overnight at 37 °C. The cultivated culture was harvested by centrifugation at 5,000 rpm for 5 min The collected pellet was resuspended in 2.5 ml of TE buffer (1M Tris-HCl, 0.5mM EDTA pH 8.0) containing 1% (w/v) SDS and washed twice with the same buffer. After washing, the pellet was re-suspended into 500 µl of prewarmed (65°C) lyses solution [(1.0M Tris-HCl (pH 8), 0.5M EDTA, 5.0M NaCl, 1.0% (v/v) β-mercaptoethanol, 20 µl SDS (10% w/v) and 5 µl proteinase K (10 mg ml^{-1})]. The cell suspension was incubated in a water bath at 65 °C for 1 h with gentle shaking and then left to cool at room temperature for 10 min. The solution was emulsified gently by equal volume of Tris saturated phenol chloroform (1:1), centrifuged at 5000 rpm for 5 min and the aqueous phase was transferred to a new tube. The aqueous solution was then washed twice by an equal volume of chloroform: isoamylalcohol, 24:1 and centrifugation at 5000 rpm for 5 min. After centrifuging, the aqueous phase removed to Eppendorf tube (400 µl/ tube) and RNA was removed by addition of 0.5 µl of RNase A and left at RT for 30 min. 2 volume of ice cooled absolute ethanol was added in presence of 10% of 5 M ammonium acetate solution and mixed gently to precipitate the DNA. The mixture

was then centrifuged at 14000 rpm (REMEI) for 10 min, the supernatant was discarded and the formed pellet was washed twice by 70% ethanol. The remained ethanol was removed by leaving the pellet to dry at room temperature. The pellet was dissolved in TE buffer [10mM Tris-HCl, 1mM EDTA (pH 8.0)].

2.3.2. PCR Amplification and sequencing of the 16S ribosomal DNA (rDNA)

Plasmid harboring lactic acid bacteria were further identified at species level by PCR amplification and sequencing of 16S rRNA gene. Amplification of 16S rRNA gene was carried out using PTC Thermocycler (Bio Rad). Primers F8-27 and R1541-1522 (Table A.1.1) (Koort *et al.*, 2004) were used to identify *E.faecium* DJ1, *E.faecium* DJ2 and primers F8-27 and 342R (Rivas *et al.*, 2004) were used to identify *E.faecium* DJ3, *Weissella confusa* NKD1, *Pediococcus pentosaceus* NS01, *Staphylococcus epidermidis* NJ1.

A standard PCR reaction (50 µl volume) was set up as follows

Template DNA	-	100 – 250 ng
10mM dNTP mix (Finnzyme)	-	200µM
Primers (Both For and Rev)	-	200 – 300 ng each
10X Taq Buffer (Genei)	-	volume to 1X final concentration
Taq DNA polymerase (Genei)	-	1 .25 units
Sterile Millipore Water	-	q. s. to make 50 µl

The PCR was done using following conditions: 95 °C, 3 min; 95 °C, 1 min; 50 °C, 50 sec; 72 °C, 1 min; 72 °C, 5 min for 30 cycles. Sequencing was performed twice on both strands by the dideoxy method using ABI 3100 DNA sequencer version 5.1.1 (Applied Biosystem). Sequence similarity search was performed in the GenBank server using the BLAST program (Altschul *et al.*, 1990). The Genbank Accession numbers of the submitted sequences are: GU358405, GU358406, GU358407, GU358408, JF734336 and JF734337 for the isolates *E. faecium* DJ1, *E. faecium* DJ2, *Weissella confusa* NKD1, *Pediococcus pentosaceous* NS01, *E. faecium* DJ3 and *Staphylococcus epidermidis* NJ1 respectively.

2.4. PLASMID ISOLATION AND CHARACTERIZATION

2.4.1. Plasmid DNA isolation from LAB

Plasmid DNA isolation from Gram-positive bacteria was performed either by the method described by Anderson and McKay (1983) or as described in Qiagen Kit protocol.

2.4.1.1. Anderson and McKay method (Anderson and Mckay, 1983)

Reagents used:

1. Sucrose buffer: 6.7 g/100 ml sucrose, 50mM Tris-Cl, 1mM EDTA pH 8.0
2. Lysozyme buffer: 25mM Tris-Cl (pH 8.00), Lysozyme (Sigma): 35 $\mu g\ ml^{-1}$
3. EDTA Tris buffer: 0.25M EDTA, 50mM Tris-Cl, pH 8.0
4. SDS buffer: 20% (w/v) SDS, 50mM Tris-Cl, 20mM EDTA
5. 3N NaOH (HI-MEDIA)
6. 2M Tris-Cl (pH 7.0)
7. 5M potassium acetate (pH 5.2)
8. Tris EDTA buffer: 10mM Tris-Cl, 1mM EDTA, pH 8.0
9. Tris-Saturated Phenol (Genei) (pH 8.0), Chloroform:Isoamyl alcohol (24:1)
10. Absolute alcohol (99.5%) and 70% ethyl alcohol (Jaipari)

LAB culture was grown in the appropriate M-17 or MRS broth at 30 °C for 16-24 hours. 4% of 16-24 hours old culture was transferred into lysis broth. Strains were propagated for 4 h at 30 °C and were harvested by centrifugation at 10000 rpm (REMEI) for 10 min. at 4°C. Pelleted cells were resuspended in 379 µl sucrose buffer and warmed to 37 °C for 5 min. To this, 150 µl lysozyme buffer was added and incubated at 37 °C for ½ an hour. The resulting solution was treated with 48.2 µl EDTA-Tris and 27.6 µl SDS buffer, mixed immediately and incubated for 5 to 10 min. for complete lysis. The lysed solution was added with 3N NaOH by intermittent mixing or swirling for 10 min. to this solution, 71.7 µl of 5M chilled potassium acetate (pH 5.5) was added. A phenol: chloroform: isoamyl alcohol (25: 24: 1) treatment was then given to remove all the proteins. DNA was then precipitated overnight by adding twice the volume of 100% ethanol in the presence of $1/10^{th}$ volume of 3M Na - acetate. After overnight precipitation, solution was centrifuged at

8000 rpm for 30 min at 4°C. Supernatant was discarded and pellet was washed with 1 ml of chilled 70 % ethanol at 10000 rpm for 15 min. The pellet was air dried and suspended in adequate amount of 1X TE

2.4.1.2. Qiagen protocol (starter culture: 500 ml)

Reagents used:

1. Buffer P2 (lysis buffer): 200mM NaOH, 1% SDS (w/v)
2. Buffer P3 (neutralization buffer): 3M potassium acetate (pH 5.5)
3. Buffer QBT (equiulibration buffer): 750mM NaCl, 50mM MOPS (pH 7.0), 15% isopropanol (v/v), 0.15% triton® X-100 (v/v)
4. Buffer QC (wash buffer): 1M NaCl, 50mM MOPS (pH 7), 15% (v/v) isopropanol
5. Buffer QF (elution buffer): 1.5M NaCl, 50mM Tris-Cl (pH 8.5), 15% (v/v) isopropanol
6. STE: 100mM NaCl, 10mM Tris-Cl (pH 8.0), 1mM EDTA
7. Lysozyme (Sisma): 10 mg ml^{-1}

LAB culture was grown in the appropriate M-17 or MRS broth at 30 °C for 16-24 hours. 4% of 16-24 hours old culture was transferred in lysis broth. Strains were propagated for 4 h at 30 °C and were harvested by centrifugation at 10000 rpm for 10 min. at 4 °C. Bacterial pellets were washed with 20 ml STE buffer and resuspended in 20 ml STE buffer containing 10 mg ml^{-1} lysozyme and incubated at 37 °C for 1.2 h. Resuspended pellet was gently mixed with 20 ml buffer P2 and incubated at room temperature for 5 min. To, this 20 ml of chilled buffer P3 was added, mixed gently and centrifuged at 12000 rpm for 30 min at 4 °C. Supernatant was removed and centrifuged again at 12000 rpm for 15 min at 4 °C. Now again supernatant was removed and collected in Oakridge tube. This supernatant was used for loading Qiagen Tip 100. Before loading, Quiagen tip 100 was equilibrated with 10 ml of QBT buffer and then it allow the above collected supernatant enter the resin by gravity flow. For washing, 2x30 ml buffer QC was applied to the sample loaded Quiagen tip. Elution of the plasmid DNA was achieved by adding 15 ml buffer QF. Eluted plasmid DNA sample was collected in the Oakridge tube and precipitated by adding 10.5 ml

(0.7 volume) room temperature isopropanol. Sample was mixed gently and centrifuge immediately at 12000 rpm for 30 min at 4 °C. Supernatant was decanted and pellet was washed with 5 ml of room temperature 70 % ethanol. The pellet was air dried and suspended in adequate amount of 1X TE

2.4.2. Cloning of pNJAKD plasmid DNA in pBluescriptSK+

2.4.2.1. Restriction digestion of vector and insert

The vector pBS SK+ was digested with *EcoR* V restriction enzyme and purified [QIAquick gel extraction kit (Qiagen)]. The insert being a pNJAkD plasmid was digested with *Pvu* II and purified with QIAquick gel extraction kit (Qiagen).

Reaction for restriction digestion of vector (pBS SK+)

Template DNA	-	500 ng
Restriction Enzyme (*EcoR* V) (NEB)	-	2 units
Buffer (10X)	-	2 µl (1X)
Sterile Millipore Water	-	Volume to make 20 µl

Reaction was incubated at 37 °C for 4 h.

Reaction for restriction digestion of insert (pNJAKD)

Template DNA	-	700 ng
Restriction Enzyme (*Pvu* II) (Fermentas)	-	5 units
Buffer (10X)	-	5 µl (1X)
Sterile Millipore Water	-	Volume to make 50 µl

Reaction was incubated at 37 °C for 6 h.

2.4.2.2. CIP treatment to Vector (pBS SK+/ Ecor V)

Template DNA (pBS SK+/ *EcoR* V)	-	375 ng
CIP buffer:	-	2µl (1X)
CIP enzyme (Fermentas):	-	2 units
Sterile Millipore Water	-	Volume to make 20 µl

Reaction was incubated at 37 °C for 30 min. Reaction was stopped by heating the mixture at 75°C for 10 min.

2.4.2.3. Ligation

The quantities of both vector and insert were calculated by measuring O. D. at 260nm. Based on the quantity calculated a reaction mix was prepared as follows:

Vector (CIP treated pBS SK+/ *EcoR* V)	-	100 ng
Insert (pNJAKD/ *Pvu* II)	-	480 ng
T4 DNA Ligase Enzyme (Fermentas)	-	5 unit
50% PEG 4000:	-	2 µl
Ligase Buffer (10X)	-	2 µl (1X)
Sterile Millipore Water	-	Volume to make 20 µl

The reaction mixture was left at 21 °C for 16 h. The enzyme was heat inactivated and the reaction product was used for transformation.

2.4.2.4. Clone Analysis

Reagents used:

Lysis Buffer:

EDTA (pH 8.0)	=	5mM
Sucrose	=	10% w/v
SDS	=	0.25%
NaOH	=	100mM
KCl	=	60mM

The colonies obtained after transformation of ligated product were first analyzed using clony lysis method. For this, the obtained individual colonies were streaked on LB Agar – antibiotic plate. This plate was kept at 37 °C for 15-16 h to get good growth of colonies. A small part of these colonies was then picked up and suspended into lysis buffer. These tubes were kept at 37 °C for 5 min, then at 4 °C for 5 min and finally at room temperature for 5 min. The tubes were centrifuged at 10,000 rpm for 5 min and the supernatant was loaded on the gel. If a clone is present in the tested colonies, a shift in the size of the plasmid bands should be seen as compared to others or the standard. This protocol thus differs from the standard plasmid isolation techniques where the plasmid appears as multiple bands (Sekar, 1987). The short-listed colonies were then grown in LB broth and plasmid was isolated using routine alkaline lysis method. The obtained plasmid is further analyzed by restriction

digestion at the multiple cloning regions of the vector and the restriction sites present in the insert. This determines a proper clone carrying only one insert in the vector.

2.4.3. DNA sequencing

Sequencing of the cloned pNJAKD plasmid was done commercially at B.G., Bangalore by the primer walking method using ABI 3100 DNA sequencer version 5.1.1 (Applied Biosystem). Universal primers (T7 & SP6) as well as primers based on sequences obtained were used. The complete pNJAKD sequence was obtained by combining sequencing results of different primer runs utilizing the overlapping regions. The genbank database and protein bank database were screened for homologies through NCBI BLAST program (Altschul *et al.*, 1990) and SWISS - PROT protein sequence databank (EMBL, Heidelberg, Germany) using the default parameter setting.

2.4.4. Identification of pNJAKD single-stranded DNA

Southern blot hybridization for the verification of ssDNA replication intermediates was performed essentially as described by te Riele *et al* (1986). Therefore, plasmids were isolated by using neutral lysis method (te Riele *et al.*, 1986) and subjected to S1 nuclease (MBI-Fermentas) treatment.

Reaction mix to treat pNJAKD plasmid with S1 nuclease was prepared as follows:

- Template DNA (pNJAKD): - 950 ng
- 5X S1 nuclease buffer: - 6 µl (1X)
- S1 nuclease (Fermentas): - 10 unit
- Sterile Millipore Water: - Volume to make 30 µl

Reaction mix was incubated at room temperature for 30 min. Reaction activity was stoped by heating at 70 °C for 10 min.

S1 nuclease treated and untreated plasmids were loaded on the agarose gel. One of the gel was subjected to denaturing conditions (50 mM NaOH, 1 mM EDTA) after electrophoresis. The plasmid DNAs were transferred from agarose gels to Hybond-N membrane (Amersham). In hybridization experiments, pNJAKD was used as a probe. HRP-Label was added by crosslinking using a non-radioactive labeling kit (Amersham ECL Direct Labelling and Detection System, RPN 3000).

2.4.4.1. Southern Blotting: Hybridization, Washes, and Development

2.4.4.1.1. Sothern blotting

Southern blotting was performed in mini trans-blot electrophoretic transfer cell at 40 V overnight. Filter paper and the nylon membrane were excised to the similar dimension of gel. The equilibration of gel and soaking of filter paper, filter pads and membrane were done in 1X TBE buffer.

Gel sandwich was prepared as follows:

- The cassette, with the gray side down was placed on the clean surface
- One pre-wetted fiber pad was placed on the gray side of the cassette
- A sheet of filter paper was placed on the fiber pad
- Equilibrated gel was placed on the filter paper and the pre-wetted membrane was placed on the gel
- Finally sandwich was completed by placing a piece of filter paper on the membrane and then last fiber pad. Continuous transfer of buffer from bottom to top, air bubble was rolled out with the help of glass rod

After closing the cassette, cassette was placed in to the tank containing transfer buffer blot was run at 40 V overnight

2.4.4.1.2. Probe labeling

10 µl sample from 10 ng μl^{-1} plasmid (linearized) was taken in a sterile 1.5 ml microcentrifuge tube. To denature plasmid DNA, sample was heated for 5 min in boiling water bath and then immediately cooled on ice for 5 min and briefly centrifuged for collecting sample contents at the bottom of tube. The resulting sample was added with 10 µl labeling reagent and mixed gently but thoroughly. To this, glutaraldehyde was added, mixed gently and incubated for 10 min at 37 °C. Labeled probe was stored at -20 °C in 30% glycerol.

2.4.4.1.3. Southern hybridization and film development

Reagents used:

1. Primary wash buffer: 2X SSC

2. Secondary wash buffer: 0.1X SSC
3. Labeled probe:
4. Detection reagent: Supplied in Amersham labeling kit
5. Developer
6. Fixer
7. Kodak autoradiography film cassette
8. Kodak film

- Blots were prehybridized with at least 0.25 ml cm^{-2} hyb solution in a thermostable plastic bag for at least 15 min at 42°C with gentle agitation.
- Following pre hybridization, labelled probe was added to hyb solution (Avoid placing it directly on the Membrane), mixed gently and incubated at 42°C with gentle agitation overnight.
- Blots were carefully transferred to the 5 ml cm^{-2} primary wash buffer
- Primary washes were performed twice at 42 °C for 20 min
- Blots were transferred to clean container, and excess of secondary wash buffer was added to it
- Secondary washes were performed twice at room temperature for 5 min with gentle agitation
- Excess secondary wash buffer from the blots were removed and blots were transferred to the fresh container.
- In the dark room, required volume of detection reagent (0.125 ml cm^{-2} detection reagent1 and 0.125ml cm^{-2} detection reagent 2) was added directly to the blots on the side carrying the DNA
- Blots were dragged along the edge of tray to remove excess detection reagent, and wraped the blots in saran wrap and rubbed gently to remove bubbles.
- Exposure to film: A Kodak film cassette was used for placing blots (DNA side up) and a sheet of autoradography film on the top of blot. Cassette was closed and film was exposed for 30 min
- Film developing: after 30 min exposure, film was removed from the cassette and droped in to developer solution for 30 s with gentle swirling or rocking. Now film was removed from developer, washed with water and transfered to fixer solution.

2.5. PROMOTER IDENTIFICATION, SCREENING & CHARACTERIZATION

2.5.1. Identification and *in-vitro* synthesis of promoters

Putative promoter fragments were identified from the plasmid pNJAKD of *E. faecium* DJ1 by BPROM (www.softberry.com) and through visual inspection (Table1). The regions coding for the putative promoters were either amplified using PCR based techniques or chemically synthesized as oligonucleotides of different sizes (Table1).

2.5.1.1. Chemical based synthesis

Complementary oligo's along with the restriction sites were commercially synthesized. Equal volumes of both complementary oligos (at equimolar concentration, 200 μg ml^{-1}) were mixed in a 1.5 ml microfuge tube. 50 μl aliquots of the mixed oligos were dispensed into 0.2 ml PCR tubes. Tubes were placed in a thermal cycler and a program was set up as follows: 95 °C for 3 min; ramp cool to 25 °C over a period of 45 min and proceeded to a storage temperature of 4 °C. Microfuge tubes were spun briefly to draw all moistures from lid and stored at -20 °C. The resulting double stranded annealed oligonucleotides were phosphorylated by T4 phospho nucleotide kinase enzyme.

2.5.1.1.1. PNK treatment to annealed oligonucleotides

Phosphorylation at 5 end of annealed oligonucleotides was carried by addition of following

Annealed product	-	25 μl
10 X PNK buffer	-	3.5 μl (1X)
1mM ATP	-	1 μl
PNK kinase	-	2 units

Samples were incubated at 37°C for ½ an hour and purified by Qiagen gel extraction kit and stored at -20°C

2.5.1.2. PCR based synthesis

Primers were designed by Primer 3 (Rozen and Skaletsky, 2000). *Pvu* II and *Nco* I restriction sites were included to the forward primers (NJAKDF1: 5 - cagctgAAAGG GGTTAGAATATATC - 3 ; NJAKDF2: 5 - cagctgAAAGGGGTTAGAATAT - 3) and reverse primers (NJAKDR1: 5 - catgccatggCGCCGAAACAGCTT - 3 ; NJAKDR2: 5 - catgccatggTTGAAATAATGC TTAGGTA- 3) respectively. The PCR reactions were carried out in Perkin Elmer 2400 PCR System (Perkin Elmer) and Peltier Thermal Cycler (Gradient and Minicycler) (MJ Research). The PCR was done under following conditions: 95 °C, 3 min; 95 °C, 1 min; 50 °C, 50 s; 72 °C, 1 min; 72 °C, 5 min for 30 cycles.

2.5.1.2.1. DNA polymerase treatment to PCR product

The resulting above amplicons were treated with T4 DNA polymerase I, to make the ends blunt

A standard DNA polymerase reaction (50 µl) in 0.2 ml PCR tube was set up as follows

Template DNA (PCR product)	-	100-150 ng
10 mM dNTP mix (Finnzyme)	-	200µM
5X T4 DNA polymerase buffer	-	volume to 1X
T4 DNA polymerase enzyme (5U/ µl)	-	1 µl
Sterile Millipore Water	-	q. s. to make 50 µl

Reaction was performed at 11°C for 30 min and enzyme was inactivated at 70 °C for 10 min, after inactivation sample was transferred to 1.5 ml microfuge tube and stored at -20 °C.

2.5.1.2.2. PNK treatment to DNP treated PCR product

DNP treated PCR product was treated with T4 phospho nucleotide kinase, to add 5 PO_4^{2-}.

PNK treatment to DNP treated PCR product was done by the addition of following

DNP treated sample:	-	50 µl
10 X PNK buffer:	-	2 µl
1mM ATP:	-	1 µl

PNK kinase - 3 units

Sterile Millipore Water - q. s. to make 70 µl

Samples were incubated at 37°C for ½ an hour and purified by QIAquick gel extraction kit (Qiagen) and stored at -20°C

2.5.2. Construction of pEGFPL$_n$ vector

The complete strategy of construction of pEGFPL$_n$ vector shown in Fig. 2.2. Each synthesized promoter was cloned in respective pEGFP vector at *Pvu* II site, resulting in plasmid pEGFPD$_n$, and which was digested with *Nco* I, generated the pEGFPL$_n$ vector. All the manipulation leading to the construction of pEGFP derivatives (pEGFPD$_n$ and pEGFPL$_n$) were performed in *E. coli* DH5α. Final construct pEGFPL$_n$ were transferred to *E.coli* DH5α, JM101 and BL21 (DE3). Subscript n represents 1 to 7 for insert AKD1 to AKD7.

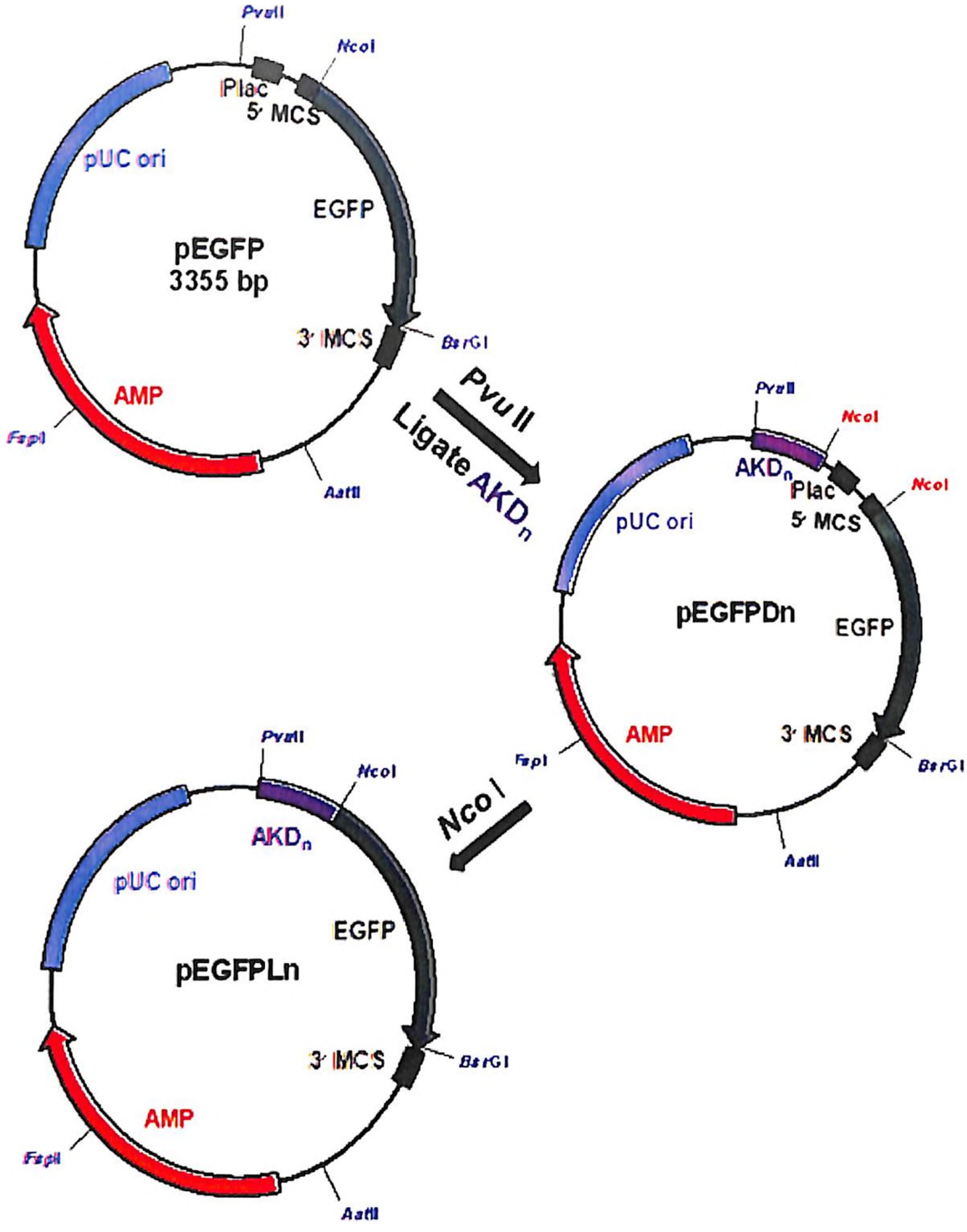


Fig. 2.2: Strategy for construction of expression vector containing eGFP gene under control with the *in-vitro* synthesized promoters (AKDn): AMP: Ampicillin resistance gene; ori: origin of replication; n = either 1, 2, 3, 4, 5, 6 or 7

2.5.2.1. Restriction digestion of pEGFP vector

Template DNA (pEGFP	-	500 ng
Restriction Enzyme (*Pvu* II)	-	2 units
Buffer (10X)	-	2µl (1X)
Sterile Millipore Water	-	Volume to make 20 µl

Reaction was incubated at 37°C for 4 hours.

2.5.2.2. CIP treatment to Vector (pEGFP / *Pvu* II)

Template DNA (pEGFP / *Pvu* II)	-	15µl
CIP buffer	-	2 µl (1X)
CIP enzyme	-	2 units
Sterile Millipore Water	-	Volume to make 20 µl

Reaction was incubated at 37°C for 30 min. reaction was stopped by heating the mixture at 75°C for 10 min

2.5.2.3. Ligation of *in-vitro* synthesized promoter to dephosphorylated pEGFP at *Pvu* II site

Blunt end ligation reaction of annealed and PCR product to the dephosphorylated pEGFP/Pvu II was carried out by the addition of following

Annealed (PNK treated) or PCR product (DNA pol and PNK treated)	- 50 - 80 ng
Dephosphorylated Vector (CIP - pEGFP/*Pvu* II)	- 60 - 150 ng
10 X T4 DNA ligase buffer	- 1.5 µl
50% PEG 6000	- 1.5 µl
T4 DNA ligase enzyme	- 5 unit
Sterile Millipore Water	q. s. to make 15 µl

Ligation samples were incubated at 24°C for 14 hours and diluted five fold in TAE buffer. 10 µl diluted samples were used for transformation in *E.coli*. The resulting construct is designated as $pEGFPD_n$.

2.5.2.4. Restriction digestion of $pEGFPD_n$ with *Nco* I

A digestion reaction was set up as follows:

Template DNA ($pEGFPD_n$)	-	600 ng
Restriction Enzyme	-	3 units
Buffer (10X) NEB	-	10µl (1X)
Sterile Millipore Water	-	Volume to make 100 µl

Reaction was incubated at 37°C for 6 hours. After digestion, the larger fragment was eluted and then a self ligation reaction was set up as follows:

$pEGFPD_n$ / Nco I	-	150ng
10 X T4 DNA ligase buffer	-	1µl (1X)
T4 DNA ligase enzyme	-	1unit
Sterile Millipore Water	-	q. s. to make 10 µl

Ligation reaction was incubated at 16 °C for overnight.Complete 10µl ligation reaction was used for transformation of *E. coli.* Transformed *E.coli* was selected on LB - AMP (100µg/ml) plate. Confirmation of clone was done by digestion with *Nco* I enzyme. This construct is designated as $pEGFPL_n$.

2.5.3. EGFP assay

EGFP assay was used for measuring the strength of *in vitro* synthesized promoters in various strains of *E.coli* such as DH5α, JM101 and BL21 (DE3). Expression of eGFP was checked at temperature change from 30 °C to 37 °C and from 30 °C to 42 °C. Samples were taken at 4, 6, 8 and 12 h of induction and analyzed. Cells were analyzed for fluorescence intensity using a $Victor^3{}_{TM}$ Multilabel Counter system (Wallac, Perkin Elmer).

a) *E.coli* cells containing $pEGFPL_n$ recombinant plasmid was grown at 30 °C for 16 hours
b) 1 ml of 16 hours old culture was transferred to 10-10ml LB broth in three 100 ml conical flasks; these flasks were marked as A, B & C.
c) Flask A was incubated at 30 °C, flask B was incubated at 37 °C and flask C was incubatedat 42 °C.

d) 300 µl samples was taken in ELISA plate (Nunc) from flask A, B & C after 4 hours incubation

e) Samples were measured for eGFP fluorescence intensity (Counts/second) by Multilabel counter system (Victor, PerkinElmer)

f) Similarly, 300 µl samples was taken in ELISA plate (Nunc) from flask A, B & C after 6, 8 and 12 hours incubation and measured for eGFP fluorescence intensity (Counts/second) by Multilabel counter system (Victor, PerkinElmer)

Chapter 3

Identification and screening of lactic acid bacteria for the presence of plasmids

Chapter 3

3.1. INTRODUCTION

Lactic acid bacteria (LAB) are a taxonomically diverse group of Gram-positive bacteria that share the property of converting fermentable carbohydrates primarily to lactic acid and thus acidify the medium in which they grow. Being aero tolerant anaerobes, the LAB family occupies a wide range of natural ecological niches. They are found on plant surfaces, among the resident microflora of the gastrointestinal tract of vertebrates, as well as in sewage, milk and soil (Chen *et al.*, 2005; Musikasang *et al.*, 2009). LAB constitute a group of Gram-positive bacteria, including *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus* as well as the more peripheral *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Teragenococcus*, *Vagococcus*, and *Weisella*; these belong to the order Lactobacillales (Wood and Holzapfel, 1998). The LAB are arguably the second only to yeast in importance in their services to mankind. They have been used worldwide in the generation of safe, storable, organoleptically pleasing food stuffs for centuries (Zhu *et al.*, 2009). These foods include fermented milk products (such as cheese, yogurt and kefir), bread and cereals (e.g., sourdough, ogi), beverages (malolactic fermentations in wines), vegetables (sauekraut, silage, kimchi) (Geis, 2003). Therefore, LAB play an important role in the food industry as a result of their fermentative capacities. Over the past decade, interest in the study of LAB has dramatically increased. This reflects not only the growing industrial importance of these bacteria for a wide range of fermentation processes but also the emergence of their application as 'probiotics', (Ali, 2010) i.e., strains to which nutritional and human/animal health beneficial properties are attributed. Therefore isolation and identification of new strains is important for industrial applications and probiotic discovery related research. Detection of plasmids in some strains of LAB, further strengthens the need to study this bacteria. The plasmids of these bacteria have been used, after genetic modification as food-grade cloning systems and for the construction of cloning and expression vectors (Shareck *et al.*, 2004; Tarakanov *et al.*,

2004; Yeng, *et al.*, 2009). The rapid progress in the genetic engineering field of lactic acid bacteria was made possible mainly due to two things (i) the identification of technologically important functions borne by plasmids (e.g. bacteriocin production, lactose utilization, casein degradation) (Teuber *et al.*, 2006) and (ii) the adaptation of *Escherichia coli* (*E. coli*)-derived technology for the isolation and manipulation of lactic acid bacteria borne plasmids. Therefore, food and pharmaceutical industry can benefit enormously from both the lactic acid bacteria and the plasmids contained in them.

Here in this chapter we report the isolation and identification of lactic acid bacteria from randomly collected water, milk, soil and plant samples. A few of these isolates have also been identified as possessing plasmids of different sizes, future molecular characterization of which will be undertaken.

3.2. RESULTS

Twenty one of the strains were identified as LAB and they were classified into the genera *Lactobacillus, Lactococcus, Leuconostoc, Enterococcus,* and *Staphylococcus* based on their morphology and physiological characters (Table 3.1).

1. Ten isolates grew at 15 °C but not at 45 °C or in 6.5% NaCl. These isolates were identified as lactobacilli according to the criteria of Kandler and Weiss (1986).
2. Three isolates grew at 15 °C, 40 °C and 45 °C, survived after heating at 60 °C after 30 min and also grew at pH 9.6. These isolates were identified as enterococci (Devriese *et al.*, 1987).
3. Five isolates, which were capable of growing at 15 °C and 40 °C, but not at 45 °C or at pH 9.6, were identified as lactococci (Mundt, 1986).
4. Two isolates which were catalase positive, were identified as staphylococci (Gotz *et al.*, 2006)
5. A oxidase positive isolates was identified as *Leuconostoc* (Sakmoto *et al.*, 1996)

Table 3.1: Morphological and physiological characteristics of LAB isolates

Isolates / Characteristics	CM1	CM2	CW	SM	CT	PP	CL1	JP	NP	AL	AM	CD	CL2	DM	MG	NM	SG	YM	AC	BM	SC
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cell shape	C	C	C	R	C	C	C	C	C	C	C	R	R	R	R	R	R	R	R	R	R
Fermentation type	H	H	H	h	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	h
Catalase test	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Oxidase test	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at or in																					
15 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
40 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45 °C	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
60 °C/30 min.	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pH 4.3	+	+	+	N	N	N	N	N	N	+	+	N	N	N	N	N	N	N	N	N	N
pH 6.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 9.6	+	+	+	-	-	-	-	-	-	N	N	-	-	-	-	-	-	-	-	-	-
4.5% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6.5% NaCl	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Identified as	Enterococci			*Leuco nostoc*	Lactococci					Staphylo cocci		Lactobacilli									

CM1: Camel milk Bikaner; CM2: Camel milk Jodhpur; CW: Cow milk; SM: Sheep milk; BM: Buffalo milk; SC: Silk cotton (*Bombax malabarica*) flower; CT: Cactus leaves; MG: Marie gold flower (*Baileya multiradiata*); CL1: Calotropis (*Calotropis gigantea*) flower; AC: Acacia flowers; AL: *Aloe vera* leaves; AM: Amla (*Phyllanthus emblica*) leaves; CD: Calendula flowers (*Calendula officinalis*); CL2: Calotropis (*Calotropis gigantea*) stem; DM: Drumstick (*Moringa pterygosperma*) flowers; PP: Poppy (*Papaver somniferum*) seeds; NM: Narmada waters; SG: Shivganga water; YM: Yamuna water; JP: Jabalpur soil; NP: Nagpur soil; H: homofermentative; h: heterofermentative; C: coccus; R: rod; N: not checked; +: positive; -: negative

The LAB isolates were then screened for the presence of plasmids. The six isolates were found to harbor the plasmids (Fig. 3.1). These plasmids are yet to be sequenced and characterized. 16S rRNA gene sequencing is an important and accurate method for bacterial identification at species level in addition to the conventional phenotypic and biochemical methods. A 350 - 1200 bp segment of the 16S rRNA gene of the LAB isolates containing plasmids were sequenced and sequences were compared to the strains in the NCBI database.

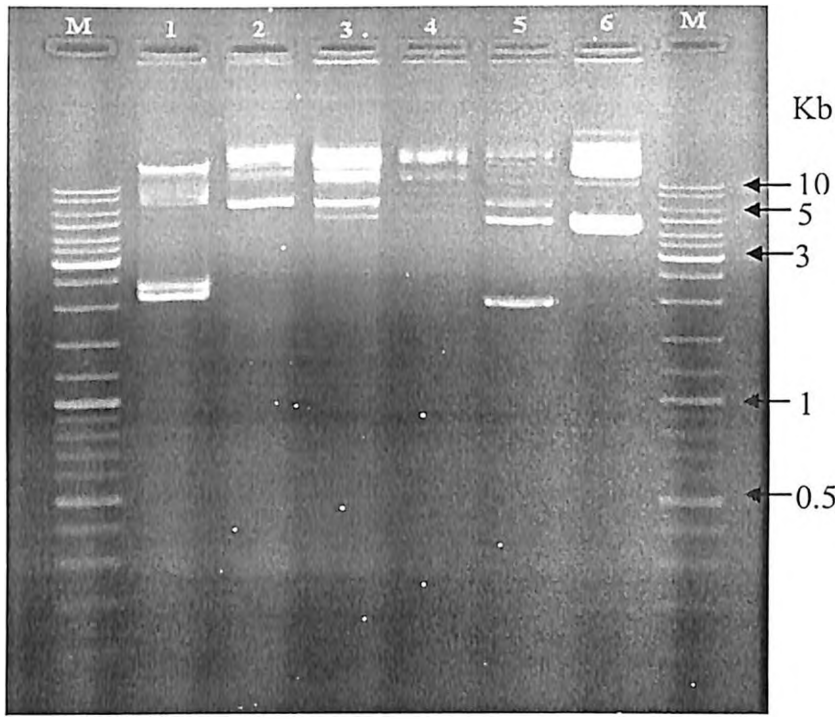


Fig. 3.1: Gel picture of crude plasmid DNA extract with traces of genomic DNA: M: DNA ladder mix MBI Fermentas (SM 0331); 1: *E. faecium* DJ1; 2: *S. epidermiditis* NJ1; 3: *E. faecium* DJ2; 4: *P. pentosaceous* NS01; 5: *E. faecium* DJ3; 6: *W. confusa* NKD1; 3.53x3.77 inch (300X300 DPI)

The differentiating characteristics of the plasmid containing isolates (CM1, CM2, CW, SC, NP, and AM) are given in Table 3.2. Each isolate showed variation in their sugar fermentation pattern, antibiotic susceptibility and 16S rDNA sequencing results. The carbohydrate fermentation was carried out for D-glucose, D-fructose, maltose, D-arabinose, ribose, glycerol, galactose, D-mannose, sucrose, cellobiose, mannintol, lactose, and sorbitol (Table. 3.2). A bright yellow color indicated the production of acid product from fermentation of sugar (Fig. 3.2). The three isolates such as CM1, CM2, and CW are able to produce acid from D-glucose, D-fructose, maltose, D-

arabinose, ribose, glycerol, D-mannose, sucrose, cellobiose, mannintol, lactose. These isolates are not fermenting sorbitol (Table 3.2). Thus the production of acid from sucrose but not from the sorbitol by the CM1, CM2, and CW isolates may be identified as *E. faecium*. The SC isolate ferments D-glucose, D-fructose, maltose, D-mannose, and sucrose and not able to ferment lactose, mannintol, sorbitol, D-arabinose, ribose, glycerol and galactose (Table 3.2). The isolate tested (SC) was found negative for acid production from arabinose and lactose. This pattern is shared by number of weissella species such as *Weissella confusa, Weissella kimchi, Weissella cibaria* (Choi *et al.*, 2002). These species were differentiated by 16S-rDNA sequencing. The NP isolate produces acid from D-glucose, D-fructose, maltose, D-arabinose, ribose and not from the sucrose, cellobiose, mannintol, and sorbitol (Table 3.2). The most strains of *Pediococcus pentosaceus* can produce acid from maltose (Barros *et al.*, 2001). Therefore, NP isolates may be identified as *Pediococcus pentosaceus*. The AM isolate ferments D-glucose, D-fructose, maltose, glycerol, galactose, D-mannose, sucrose, and lactose. This isolate was not able to ferment arabinose, ribose, cellobiose, mannintol, and sorbitol. Most of the *Staphylococcus species* are not able to ferment mannintol except *Staphylococcus epidermidis* (Geary *et al.*, 1989). Therefore, the AM isolate may be *Staphylococcus epidermidis.*

The plasmid containing isolates were screened for the resistance to various antibiotics, such as ampicillin, tetracycline, vancomycin, chloramphenicol, erythromycin and kanamycin. Antibiotic resistant LAB isolates change the purple color of MRS-BCP plate to yellow (Fig. 3.3). The isolates *E. faecium* DJ1, *E. faecium* DJ2 and *W.confusa* NKD1 were found to be vancomycin resistant and *E. faecium* DJ3 was vancomycin and tetracycline resistant (Table 3.2). Toomey et al. (2010) found that *E. faecalis* strains isolated from Irish pork and beef abattoirs were susceptible to vancomycin, however, 4 of 10 strains of *E. faecium* were resistant to vancomycin but no corresponding genetic determinants for this phenotype were detected. Johnston *et al.* (2004) observed the tetracycline and vancomycin resistant *E. faecium.* Olano *et al.* (2001) reported the presence of vancomycin resistant *Weissella confusa* strain in blood culture. These reports support our species identification results. An isolate *Pediococcus pentosaceus* is resistant to kanamycin (Table 3.2). This result is supported by a work of Bacha *et al* (2010).

To confirm the phenotypic and biochemical identification of CW, CM1, CM2, NS, AM, and SC isolates, definitive species identification was done on the basis of sequence data analysis for the 16S-rDNA. The NCBI-Blast analysis of the 16S-rRNA gene of CW (1392 bases), CM1 (801 bases), and CM2 (450 bases) isolates showed the maximum identity (99%) to that of the *E. faecium* (most strains). The 16S-rRNA gene of SC (343 bases) and AM (336 bases) isolates showed 98% similarity with most of the strains of *Weissella confusa* and *Staphylococcus epidermidis* respectively. The 16S-rRNA gene of NP (366 bases) isolates showed 99% similarity with *Pediococcus pentosaceus* (Most strains). These identified species were compared with their close relatives by multiple sequence alignment of 16S-rRNA genes (Fig. 3.4 to Fig. 3.9). The 16S-rRNA genes of CW, CM1 and CM2 isolates were individually compared with the 16S-rRNA genes of *E. faecium* (FJ378708.2), *E. durans* (GQ42148.1) and *E. faecalis* (JF772057.1). Similarly the 16S-rRNA gene of SC isolate was compared to that of *W. confusa* (AB671569.1), *W. cibaria* (JQ805714.1) and *W. kimchi* (AF312874.1). The 16S-rRNA gene of NP isolate was compared to that of *P. pentosaceus* (AB550295.1), *P. stilesii* (AB621963.1), *P. parvulus* (GQ923889.1) and *P. acidilactici* (FJ917739.1). Finally the 16S-rRNA gene of AM isolate was compared to that of *S. epidermidis* (JQ795860.1), *S. capitis* (AB009937.1) and *S. aureus* (AY126148).

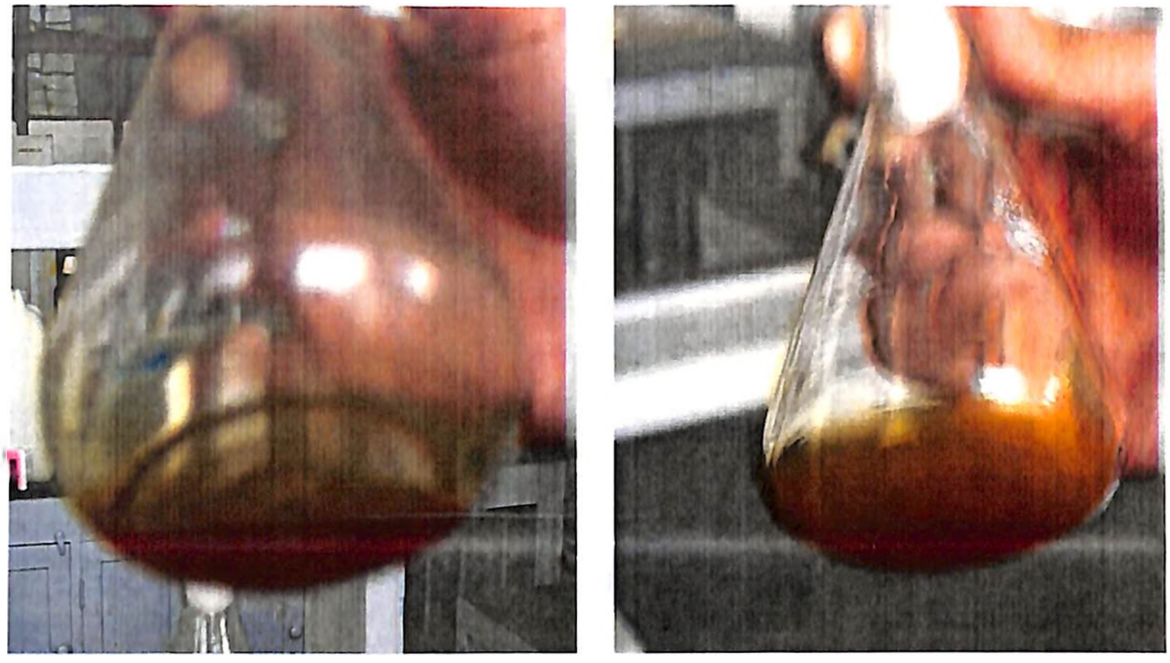
The aligned sequence clearly indicates that CM1, CM2 and CW are very-2 close to *E. faecium*, SC is close to *W. confusa*, NS is close to *P. pentosaceus*, and AM is close to *Staphylococcus epidermidis*. The 16S-rRNA gene sequence of CM1 isolate differ for 0 bases from *E. faecium* (FJ378708.2), 8 bases from *E. durans* (GQ42148.1) and 23 bases from *E. faecalis* (JF772057.1) (Fig. 3.4). The 16S-rRNA gene sequence of CM2 isolate differ for 2 bases from *E. faecium* (FJ378708.2), 6 bases from *E. durans* (GQ42148.1) and 30 bases from *E. faecalis* (JF772057.1) (Fig. 3.5). The 16S-rRNA gene sequence of CW isolate differs for 1 base from *E. faecium* (FJ378708.2), 3 bases from *E. durans* (GQ42148.1) and 38 bases from *E. faecalis* (JF772057.1) (Fig. 3.6). The 16S-rRNA gene sequence of SC isolate differs for 0 base from *W. confusa* (AB671569.1) and 8 bases from *W. cibaria* (JQ805714.1) and *W. kimchi* (AF312874.1) (Fig. 3.7). The 16S-rRNA gene sequence of NS isolate differs for 2 bases from *P. pentosaceus* (AB550295.1), 5 bases from *P. acidilactici* (FJ917739.1), 9 bases from *P. stilesii* (AB621963.1) and 26 bases from *P. parvulus* (GQ923889.1)

(Fig. 3.8). Finally the 16S-rRNA gene sequence of AM isolate differs for 2 bases from *S. epidermidis*(JQ795860.1), 8 bases from *S. capitis* (AB009937.1) and 107 bases from *S. aureus* (AY126148) (Fig. 3.9).

Table 3.2: Carbohydrate fermentation*, 16S-rDNA sequence similarity and antibiotic profiles of plasmid containing LAB isolates

Isolates →	CM1	CM2	CW	SC	NP	AM
Carbohydrates						
D-Glucose	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
D-Arabinose	+	+	+	-	(+)	-
Ribose	+	+	+	-	+	-
Glycerol	+	+	+	-	N	+
Galactose	N	N	N	-	N	+
D-Mannose	+	+	+	+	N	+
Sucrose	+	+	+	+	-	+
Cellobiose	+	+	+	N	-	-
Mannintol	+	+	+	-	-	-
Lactose	+	+	+	-	N	+
Sorbitol	-	-	-	-	-	-
Antibiotics						
Ampicillin	-	-	-	-	-	-
Tetracyclin	-	+ (20)	-	-	-	-
Vancomycin	+ (25)	+ (25)	+ (50)	+ (50)	-	-
Chloramphenicol	-	-	-	-	-	-
Erythromycin	-	-	-	-	-	-
Kanamycin	-	-	-	-	+ (25)	-
16S-rDNA sequence similarity	99% S with *E. faecium*	99% S with *E. faecium*	99% S with *E. faecium*	98% S with *W. confusa*	99% S with *P. pentosaceus*	98% S with *S. epidermidis*
Strain name submitted to GenBANK	DJ2	DJ3	DJ1	NKD1	NS01	NJ1

CM1: Camel milk Bikaner; CM2: Camel milk Jodhpur; CW: Cow milk; SC: Silk cotton flower; NP: Nagpur soil; AM: Amla leaves; digit in the small bracket [(20), (25), (50)] are the antibiotic concenteration (in µg/ ml); + growth was obserbed; - no growth; (+) weak growth; N not checked; S similarity; * Readings were taken under anaerobic conditions at 30 °C for 48 hours



Negative Fermentation **Positive Fermentation**

Fig. 3.2: Carbohydrate fermentation: Yellow colour is produced due to production of acid. Carbohydrate is metabolized by bacteria and produces acid

Antibiotic sensitive **Antibiotic Resistant**

Fig. 3.3: Antibiotic susceptibility: presence of antibiotic resistant lactic acid bacteria converts the purple color of BCP to yellow; Contol plate (plate withought antibiotic) is not shown here

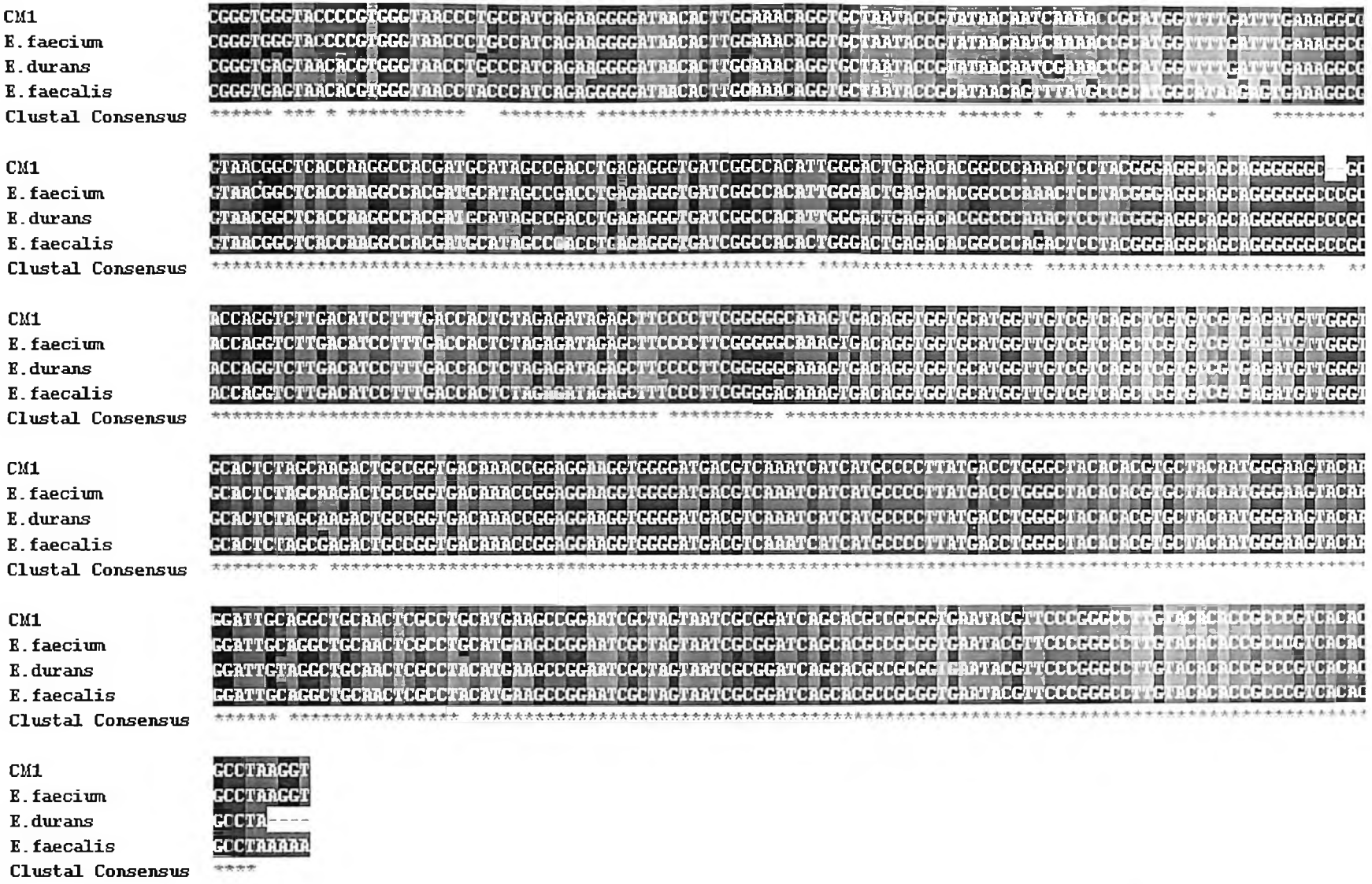


Fig. 3.4: Multiple sequence alignment of 16S rDNA sequence of Camel milk, Bikaner isolate (CM1) to that of closely related *Enterococcus* species: sequences were aligned by using EMBL-CLUSTALW2 program. Editing in sequences was done by BioEdit software.

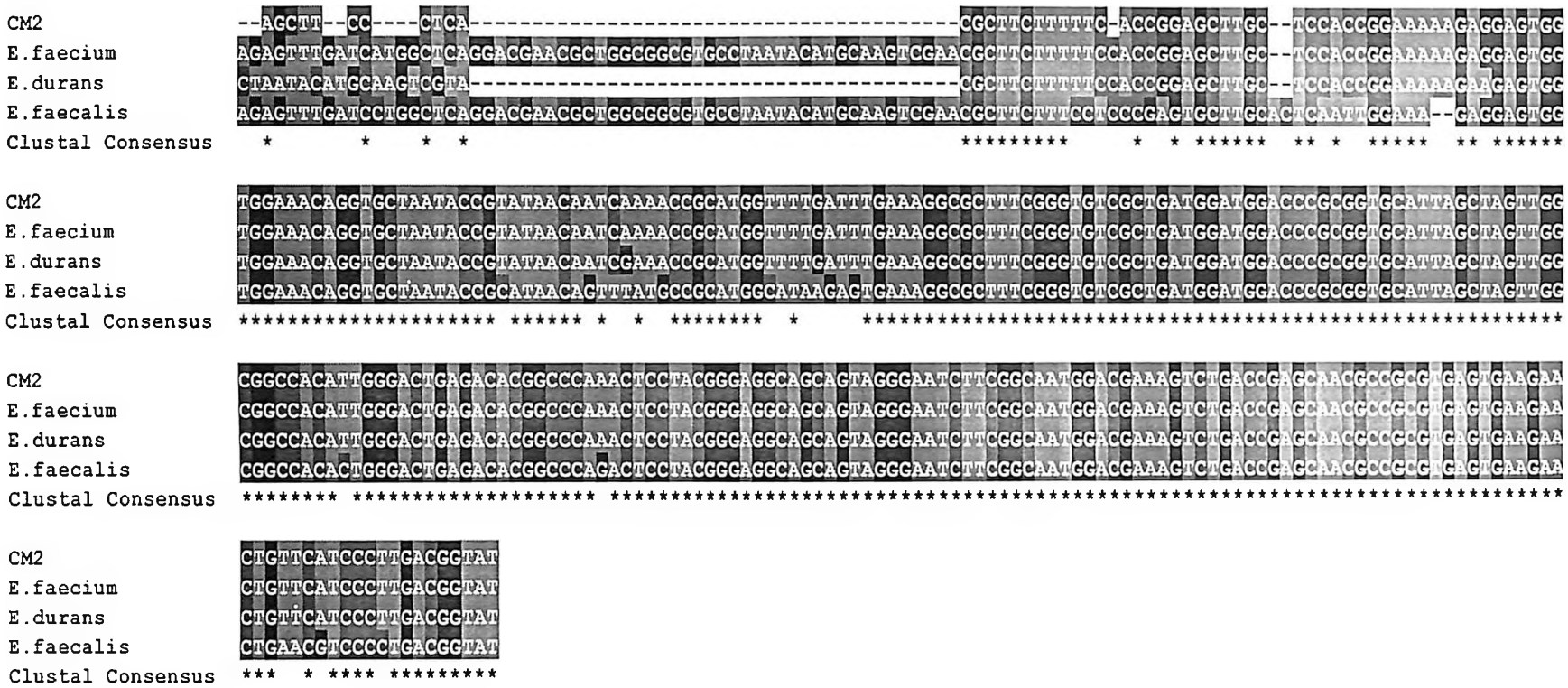


Fig. 3.5: Multiple sequence alignment of 16S rDNA sequence of Camel milk, Jodhpur isolate (CM2) to that of closely related *Enterococcus* species: sequences were aligned by using EMBL-CLUSTALW2 program. Editing in sequences was done by BioEdit software.

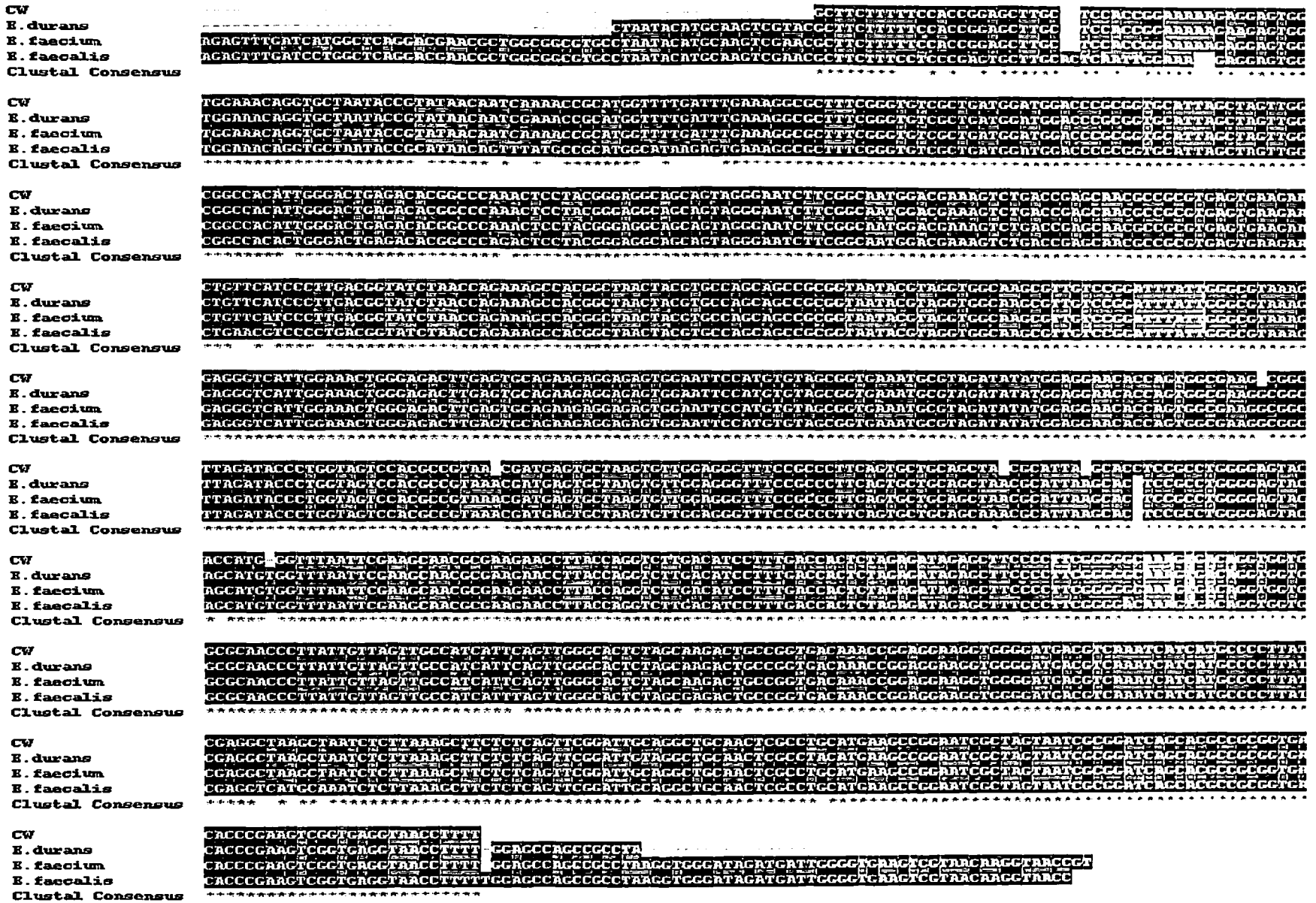


Fig. 3.6: Multiple sequence alignment of 16S rDNA sequence of Cow milk isolate (CW) to that of closely related *Enterococcus* species: sequences were aligned by using EMBL-CLUSTALW2 program. Editing in sequences was done by BioEdit software.

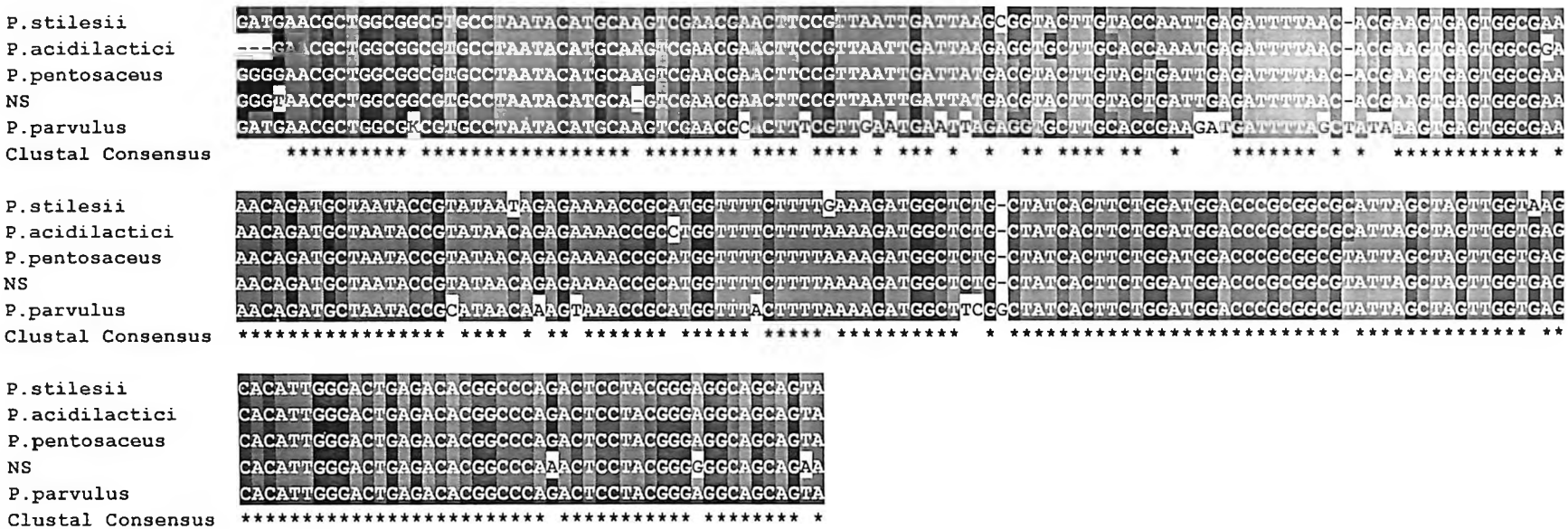


Fig. 3.8: Multiple sequence alignment of 16S rDNA sequence of Nagpur soil isolate (NS) to that of closely related *Pediococcus* species: sequences were aligned by using EMBL-CLUSTALW2 program. Editing in sequences was done by BioEdit software.

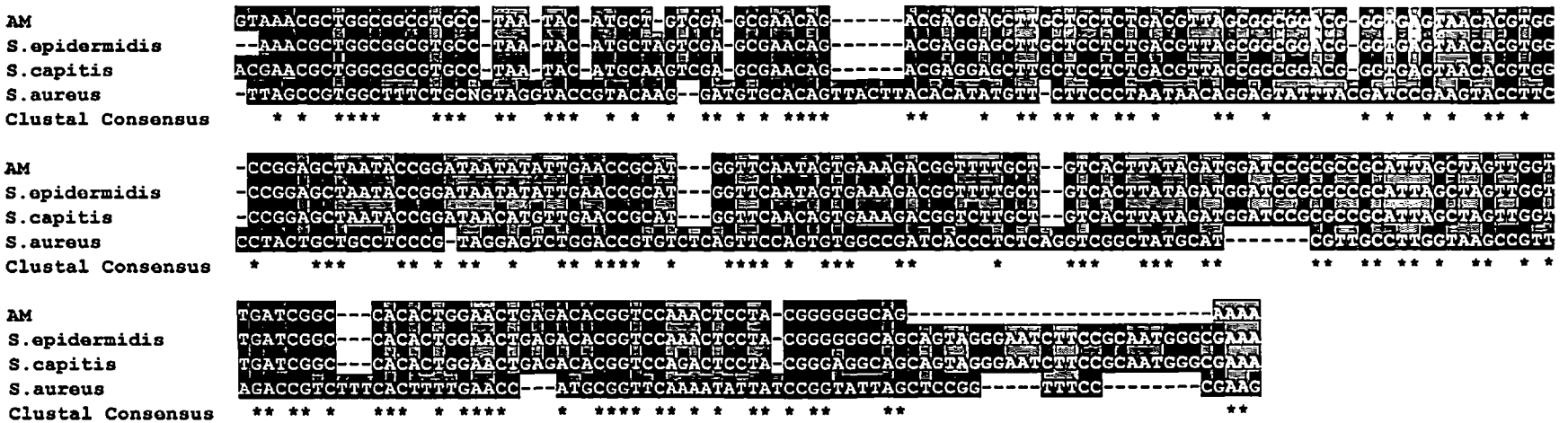


Fig. 3.9: Multiple sequence alignment of 16S rDNA sequence of Amla leaf isolate (AM) to that of closely related *Staphylococcus* species: sequences were aligned by using EMBL-CLUSTALW2 program. Editing in sequences was done by BioEdit software.

An agar well diffusion method was used to screen the plasmid containing isolates (CW, CM1, CM2, SC, NS, and AM) for antimicrobial activity against the pathogenic microbes such as *Bacillus cereus, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas putida, Escherichia coli,* and *Salmonella* sp (Table 3.3). Out of the 6 isolates mentioned, only three isolates *E. faecium* DJ1 (CW), *E. faecium* DJ2 (CM1) and *W. confusa* (SC) exhibited antimicrobial activity. The *E. faecium* DJ1 inhibits the growth of *Salmonella* spp. (d = 15.5 mm) and *S. aureus* (d = 13.2 mm). The *E. faecium* DJ2 inhibits the growth of *S. aureus* (d = 11 mm). The *Weissella confusa* NKD1 inhibits the growth of *E. coli* (d = 9.5 mm).

Table 3.3: Antimicrobial effects of the supernatants of lactic acid bacteria

Tested LAB	Inhibition of indicator strains Diameter of inhibition (mm)*					
	Bacillus cereus	*Staphylococcus aureus*	*Klebsiella pneumonia*	*Pseudomonas putida*	*Escherichia coli*	*Salmonella* sp.
E. faecium DJ1	0	13.2	0	0	0	15.5
E. faecium DJ2	0	11	0	0	0	0
E. faecium DJ3	0	0	0	0	0	0
S. epidermidis NJ1	0	0	0	0	0	0
W. confusa NKD1	0	0	0	0	9.5	0
P. pentosaceus NS01	0	0	0	0	0	0

* Diameter of clear zones around the wells excluding well (6 mm); 0 No inhibition

3.3 DISCUSSION

The studies on LAB detail their use for probiotic purposes and as oral vaccines. Most probiotic microorganisms grouped under LAB are from the *Lactobacillus* sp., *Bifidobacterium* sp. and *Enterococcus* sp. (Klein *et al.*, 1998; Roberfroid, 2000). The identification of species of LAB cannot however be limited only to purely morphological, physiological or biochemical analysis of the bacteria. Interestingly we have found that the NP and SC isolates were identified as *Lactococcus* and *Lactobacillus* respectively by phenotypic identification scheme. Later these isolates were identified as *Pediococcus* and *Weissella* by biochemical and 16S-rDNA sequencing schemes. *Weissella* and *Pediococcus* can be confused with the *Lactobacillus* and *Lactococcus* respectively. The morphology and physiology of the *Weissella* are similar to lactobacillus. *Weissella* is also known as heterofermentative *Lactobacillus*. Collins *et al.* (1993) classified five heterofermentative species of *lactobacillus* to new genus *Weissella* by biochemical identification test. Similarly the pediococci can be wrongly interpreted as cocci shaped lactococci, when they exist as chain form. They can be distinguished by biochemical tests. *E. faecium*, *E. durans* and *E. faecalis* are very closely related species. They can be differentiated by the fermentation profile. *E. faecium* is differentiated from *E. faecalis* by the failure of the former to form acid in sorbitol broth. *E. faecium* is differentiated from *E. durans* by the failure of *E. durans* to form acid in sucrose. Thus the production of acid from sucrose but not from the sorbitol by the CM1, CM2, and CW isolates may be identified as *E. faecium.* The carbohydrate fermentation pattern of *Pediococcus pentosaceus* is different from its closely related species including *Pediococcus pentosaceus, Pediococcus acidilactici,* and *Pediococcus parvulus*. The most strains of *Pediococcus pentosaceus* can produce acid from maltose, whereas the *Pediococcus acidilactici,* and *Pediococcus parvulus* are not able to ferment maltose (Barros *et al.*, 2001). It is report that, mainly two species of *pediococcus* are able to ferment arabinose. They are *Pediococcus pentosaceus* and *Pediococcus acidilactici* (Barros *et al.*, 2001). The acid production from maltose by *Pediococcus sp.* clearly indicates that the NP isolates may be *Pediococcus pentosaceus*. Mannintol fermentation by staphylococci sp. is the characteristic feature of the identification of *Staphylococcus epidermidis.* Most of the *Staphylococcus species* are not able to ferment mannintol

except *Staphylococcus epidermidis* (Geary *et al.*, 1989). Therefore, the AM isolate may be *Staphylococcus epidermidis.*

The *Weissella* group was identified down to the species level through the production of acid from glucose, fructose, maltose, arabinose, galactose, sucrose, cellobiose, lactose and ribose (Bjorkroth *et al.*, 2002; Collins *et al.*, 1992). The isolate tested (SC) was found negative for acid production from arabinose and lactose. This pattern is shared by number of weissella species such as *Weissella confusa, Weissella kimchi, Weissella cibaria* (Choi *et al.*, 2002) SC isolate couldnot be identified biochemically at species level. The 16S-rDNA sequencing analysis can differentiate these species.

Indeed, two species which appear to be closely related based on morphological, physiological, or biochemical data may prone to be distantly related based on information from relevant genetic analysis. Analysis of the 16S rDNA of the LAB is thus of vital importance in determining definitively if a Lactic acid bacterium belongs to a known genus or species (Ennahar *et al.*, 2003). The 6 plasmid containing isolates (CM1, CM2, CW, SC, NP, and AM) were identified at species level by 16S rRNA gene sequences analysis. Further antibacterial activities of above isolates were performed against *B. cereus*, *S. aureus*, *K. pneumonia*, *P. putida*, *E. coli*, *Salmonella* sp. Out of the 6 isolates mentioned, only three isolates *E. faecium* DJ1 (CW), *E. faecium* DJ2 (CM1) and *W. confusa* (SC) exhibited antimicrobial activity. There are reports on antimicrobial activity of *E. faecium* against many pathogenic bacteria such as *Salmonella* spp, *S. aureus*, *E.coli* etc. (Dimov, 2007; Herranz *et al.*, 2001; Levkut *et al.*, 2009). Similar results have been observed with our isolates of *E. faecium*. *E. faecium* DJ1 inhibits the growth of *Salmonella* spp. and *S. aureus* and *E. faecium* DJ2 inhibits the growth of *S. aureus*. Interestingly, we also detected antimicrobial activity of *W. confusa* against *E. coli.* Like other bacteria, LAB can be antibiotic resistant and this might pose problems for certain usages. LAB themselves are not pathogenic but they can transfer antibiotic resistance genes to pathogenic bacteria that infect humans or animals. The presence of antimicrobial activity in one of the isolates *E. faecium* DJ1 may imply future usage in food and feed preservation related areas. Most lactic acid bacteria (LAB) are used in the production and preservation of food and feed. The major antimicrobial and biopreservative substance produced by LAB is organic acid; however, some LAB produces additional antimicrobial compounds. Among these, the bacteriocins have great potential as food preservatives (Nes and Johnsborg, 2004).

Additionally, antimicrobial compounds (phenyllactic acid (PLA), 2-hydroxy-4-methylpentanoic acid) different from the bacteriocins have recently been identified display strong antifungal activity (Ndagano *et al.*, 2011).

E.coli based plasmids for cloning and expression vector construction is predominant in scientific approaches for a number of reasons. There is comparatively less work on LAB based plasmids (Shareck *et al.*, 2004). The broad host range replication capacity and the presence of good promoter (discussed in promoter characterization chapter) in LAB plasmid make them suitable candidate for construction of expression vector. Therefore, finding novel plasmids in Lactic acid bacteria could be of significant importance for biotechnologists and genetic engineers. TThe 6 LAB isolates (*E. faecium* DJ1, *E. faecium* DJ2, *E. faecium* DJ3, *P. pentosaceous*, *S. epidermiditis* and *W. confusa*), completely characterized harbor both large and small plasmids which are yet to be characterized completely. To the best of our knowledge, the presence of plasmids in *W. confusa* has not been reported as yet in literature.

Chapter 4

Characterization of pNJAKD plasmid

Chapter 4

4.1. INTRODUCTION

E. faecium is a Gram-positive, coccoid bacteria that colonize in groups or chains. They are naturally found as a part of the digestive tract flora in many organisms, including humans and are robust microbes which are able to tolerate relatively high salt and acid concentration. The study of small plasmids from a variety of bacteria has recently become a focus of research interest and this work has led to the development of families of vectors designed for specific purposes. The genetic organization and mode of replication of a number of small cryptic plasmids from some lactic acid bacteria have been described (Jeong *et al.*, 2007; Mills *et al.*, 2006) and it is now known that these replicons are broadly similar to those from other Gram-positive (Gruss and Ehriich, 1989) and Gram-negative (Yang and McFadden, 1993) bacteria which replicate via single stranded intermediates. They are collectively referred to as rolling circle plasmids. Three classes of plasmids are known to be capable of replication in the *Enterococci*, the rolling circle replication (RCR) plasmids, the Inc plasmids, and the pheromone responsive plasmids (Weaver *et al.*, 2002). All RCR plasmids (Khan, 1997; Sanchez and Mayo, 2003) harbor a gene encoding a plasmid replication initiation protein; its target site termed the *dso*, and a specific sequence known as *sso* which make possible the conversion of SS DNA intermediates into DS plasmid. Another module often present in RCR plasmids is a mobilization entity that enables plasmid transmission between bacteria during conjugation (Guzman and Espinosa, 1997). This entity comprises two structural elements (I) *mob* - gene, which encodes Mob protein (II) the cis-element (*ori*T), Cleavage site for Mob protein (Clark and Warren, 1979). As pointed out by Ilyina and Koonin (1992), the organization and activity (DNA nicking and strand displacement) of the plasmid transfer module are reminiscent of those of the minimal replicon, with the mobilization protein (Mob) and origin of transfer (*ori*T) corresponding to the Rep and *dso* respectively. This mobilization module is, however, not restricted to RCR elements, but is also present in θ-replicating plasmids.

Here, we detail the complete nucleotide sequence of small size (3.28 Kb) plasmid pNJAKD from *E. faecium* DJ1 strain. The genetic organization and mode of replication of this plasmid is detailed, and its homology with other plasmids is examined.

4.2. RESULTS

4.2.1. Identification of genus and species of pure culture of milk isolate

16S rRNA gene sequencing and BLAST homology search (98% identity to the subject sequence) indicates that the unknown coccoid milk isolates represent a genus *Enterococcus* and species *faecium*.

4.2.2. Plamid DNA isolation, Restriction digestion and Cloning

The *E. faecium* plasmid DNA was checked on 1% agarose gel and, three bands were obtained indicating the presence of three different forms. The upper two bands are probably the multimers (M), the open circular (OC) forms, and the lower band, the supercoiled (SC) form of the plasmid DNA (Fig. 4.1).

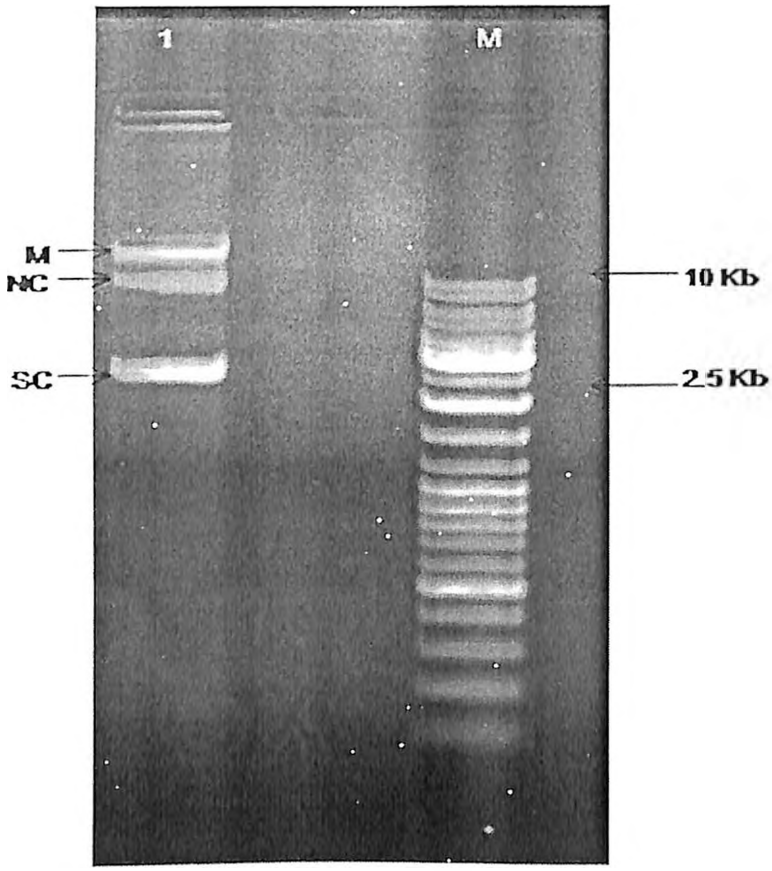
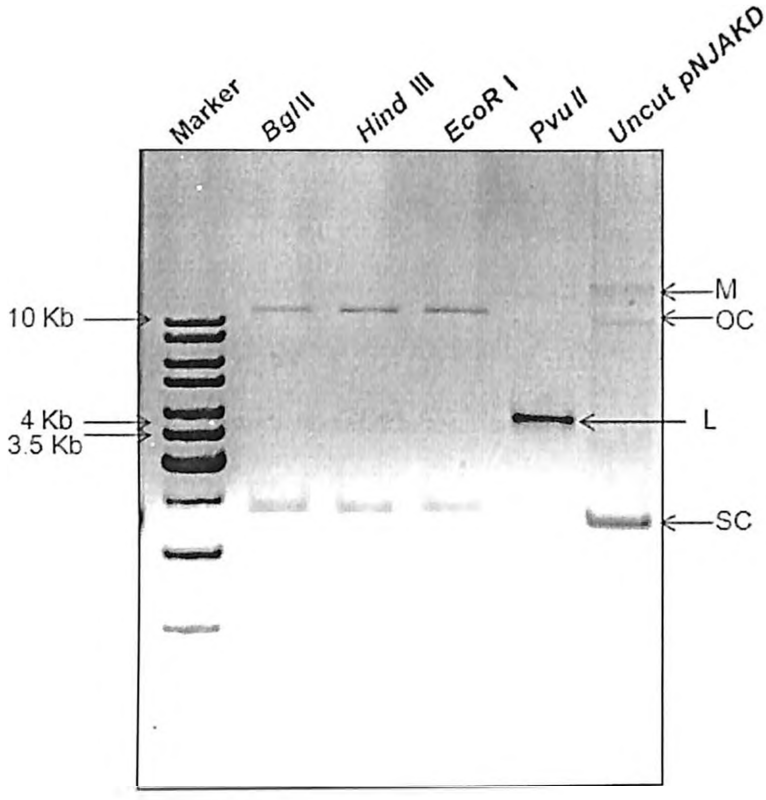


Fig. 4.1: Agarose gel electrophoresis of the pNJAKD plasmid extracted from *E.faecium* DJ1 (lane1); Plasmid DNA from *E. faecium* DJ1 was extracted from modified alkaline lysis method and subjected to agarose gel electrophoresis in 1% (w/v) agarose. M: DNA ladder mix marker MBI Fermentas (SM 03331); L: linear; OC: open circular; CCC: covalently closed circular; 4.43x3.88 inch (300X300 DPI)

The restriction enzyme *Pvu* II was used to linearize the plasmid and estimate its size. Upon restriction digestion a 4 Kb band was observed on 1% agarose gel (Fig. 4.2). There were no other bands of lower molecular weight seen in 1% agarose as well as 12% DNA PAGE. This indicated that pNJAKD has a single *Pvu* II site. The digested plasmid was then cloned in pBS SK+ at the *EcoR* V site (Fig. 4.3). The Restriction digestion of pBS-SK+ plasmid with *EcoR* V generates an approximately 4 Kb band on 1% agarose gel.



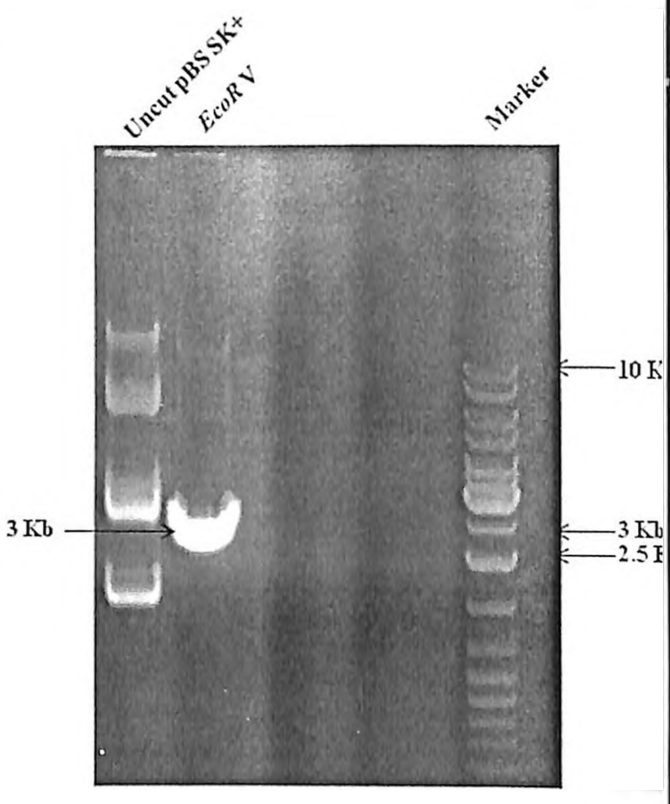


Fig. 4.2: Restriction digestion of pNJAKD plasmid DNA purified with Qiagen midi kit Marker: DNA ladder mix marker MBI Fermentas (SM 03331); The plasmid pNJAKD DNA was digested with *Bgl* II, *Hind* III, *EcoR* I & *Pvu* II; Samples were loaded on 1% agarose gel; M; multimers; OC: open circular; SC: supercoiled; L: linearized Pnjakd; The gel image has been converted to white background for proper visualization 4.13x4.05 inch (300X300 DPI)

Fig.4.3: Restriction digestion of pBS SK+ plasmid: The plasmid pBS SK+ was digested with *EcoR* V; Marker: DNA ladder mix MBI Fermentas SM 0331; 3.95x3.57 inch (300X300 DPI)

The clone was confirmed by single (*Hind* III) and double digestion (*Hind* III & *EcoR* I). After single digestion (*Hind* III), band was observed at approximately 7 Kb (size of vector+insert) and after double digestion (*Hind* III & *EcoR* I), band was observed at approximately 4 Kb (size of insert) and at approximately 3 Kb (size of vector) (Fig. 4.4). The resulting construct was used for sequencing.

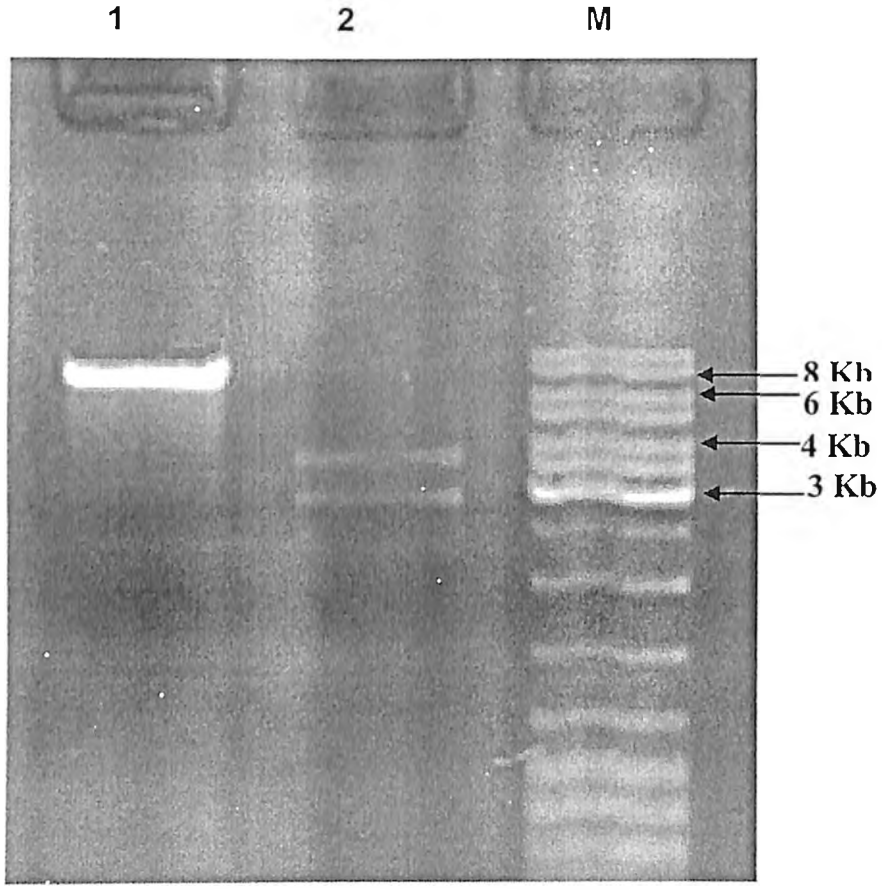


Fig.4.4: Confirmation of clone: 1: recombinant plasmid digested with *Hind* III; 2: recombinant plasmid digested with *Hind* III & *EcoR* I M: DNA ladder mix marker MBI Fermentas SM 0331;
4.17x3.77 inch (300X300 DPI)

4.2.3. Sequence determination of pNJAKD

In order to investigate the structural organization of pNJAKD plasmid in more details, the sequencing reactions were performed by using both universal primers and primers based on sequences obtained. The complete pNJAKD sequence was obtained by combining sequencing results of different primer runs utilizing the overlapping regions. The whole pNJAKD sequence consisted of 3825 bp, and was arbitrarily numbered, starting at first C of unique *Pvu* II site (Fig. 4.5).

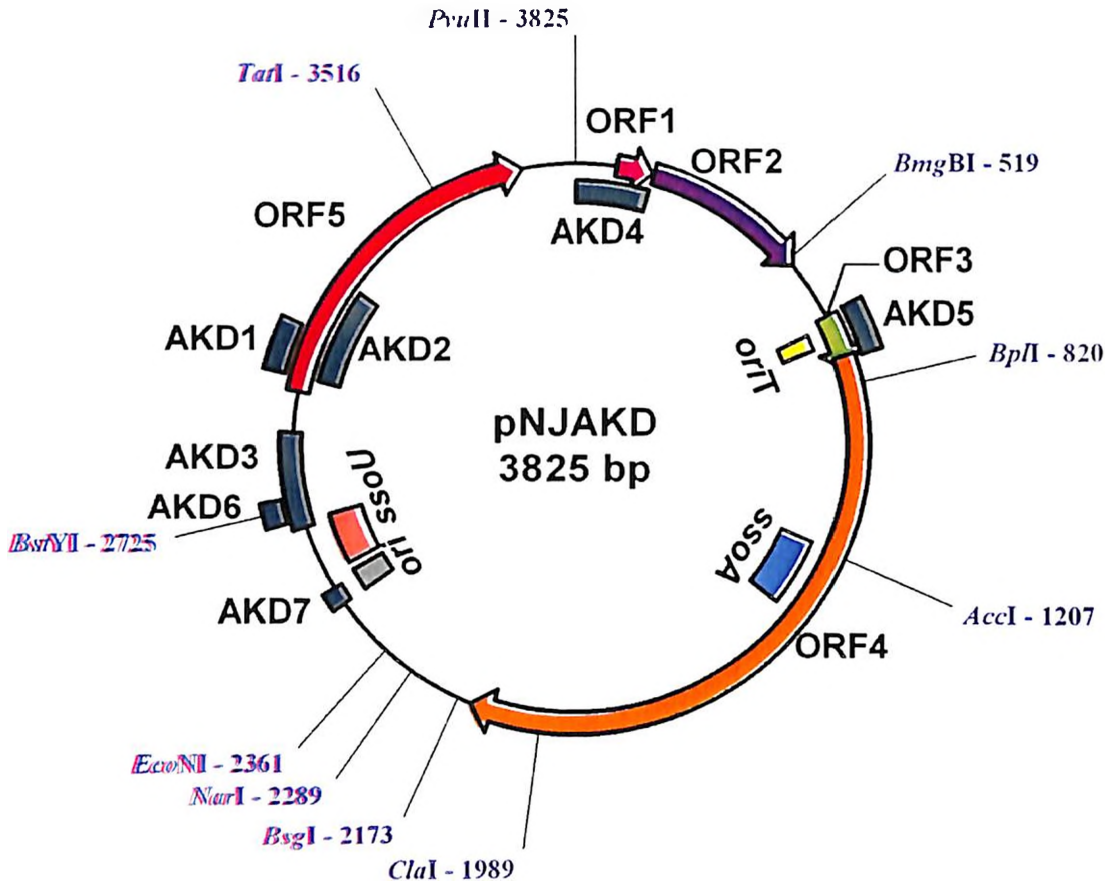


Fig. 4.5: **Plasmid map of pNJAKD:** The first C in the unique PvuII site (CAGCTG) is designated as nucleotide number1. This map summarizes relevant restriction sites, location of features derived from sequence analysis: open reading frames (ORFs), location of putative double strand origin (*ori*), single stranded origins (*ssoA, ssoU*), and origin of transfer (*ori*T) and promoters; 4.46x5.33 inch (300X300 DPI)

The % G+ C content of pNJAKD is 39.6 which is in the normal range of *Lactoccocal* RCR plasmids (Kilpper-Balz *et al.*, 1982; Schleifer and Kilpper-Balz 1987). Analysis of the sequence indicated presence of five putative open reading frames (ORFs) starting from ATG and TTG and seven promoter like regions.

4.2.4. Detection of ssDNA intermediates

The ssDNA intermediate is a hallmark of the RCR mechanism (Khan, 1997). Therefore, detection of ssDNA by Southern hybridization is routinely used to confirm the presence of plasmid replication mechanism. S1 nuclease treated and untreated plasmid DNA was transferred to nylon membranes with or without prior denaturation. When plasmid was transferred to the membrane under denaturing (alkaline) condition, all the conformational structures (a & b) were observed in the S1 nuclease untreated plasmid sample (Fig.4.6A, lane1), while band 'c' disappeared in the S1 nuclease treated sample (Fig.4.6A, lane2), indicating the presence of the ssDNA intermediate. Additionally, when the plasmid was transferred under non-denaturing conditions to the membrane, only one band (c) was detected in the S1 nuclease untreated plasmid sample. This band is in the similar position as that which disappeared when the plasmid was treated with S1 nuclease (Fig. 4.6B, lane 1). These results strongly indicate that pNJAKD undergoes replication via the RCR mechanism in the host system used.

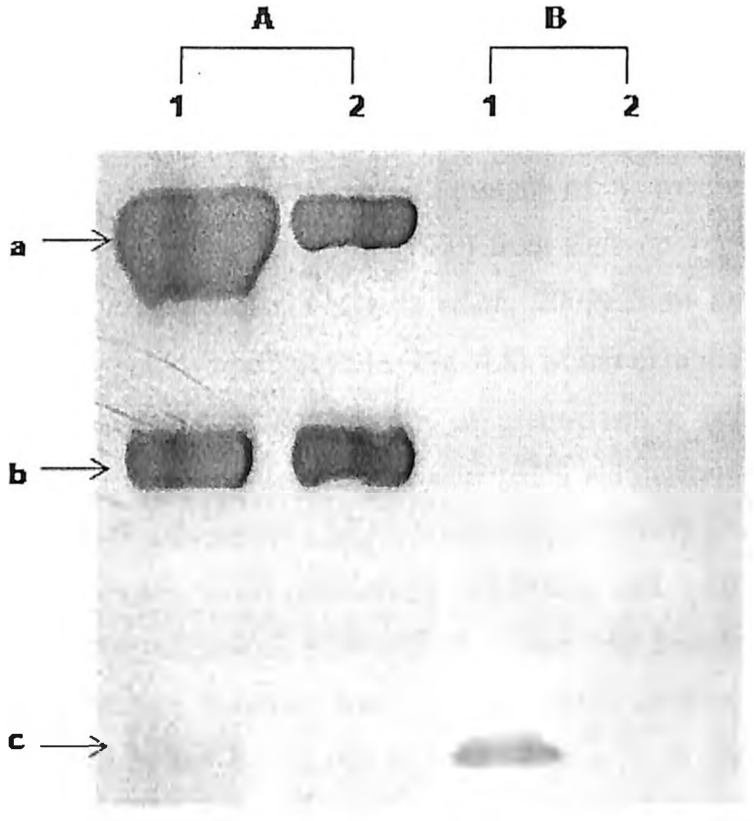


Fig. 4.6: Analysis of SS DNA production: The blots were made from denatured gel (A) and a non denatured gel (B). Plasmid isolated from neutral lysis of *E. faecium* DJ1 cells, were loaded on agarose gel without (lane1) and with (lane2) nuclease S1 treatment. a: open circular form; b: supercoiled form; c: single-stranded DNA; 4.2x3.78 inch (300X300 DPI)

4.2.5. Comparative analysis of putative polypeptide coding ORFs.

The putative polypeptides encoded by five putative ORFs of plasmid pNJAKD were screened for homology with other proteins using NCBI and SWISS-PROT (EMBL) databases. No significant similarity was observed for the putative polypeptide encoded by ORF1. ORF2 and ORF3 which code for 120 amino acids (13910 D, PI: 5.49) and 53 amino acids (6173 D, PI: 10.36) long peptides, these shows homology with predicted hypothetical proteins CDS2 (99% similarity) and CDS1 (80% similarity) respectively of the *E. faecium* plasmid pRI1 (Garcia-Migura *et al.,* 2009). Two major open reading frames (ORF4 & ORF5) with a good coding probability are located on the same strand and cover 80% of the total sequence. The larger open reading frame (ORF4) encodes a putative polypeptide (Fig. 4.7) which exhibits sequence similarity with the "Relaxase" (Mob) protein of a number of Gram-positive plasmids including pJS42 (91% similarity, B0LSL0) from *Enterococcus faecium* JH95 and pRI1(89% similarity, BOZBG6) (Gracia-Migura *et al.,* 2009) from *Enterococcus faecium* AHA15. ORF5 encodes a putative polypeptide (Fig. 4.8) which exhibits sequence similarity with the replication proteins of RCR plasmids of gram-positive bacteria, included the replication protein of pC194 (*Staphylococcus aureus* [61% similarity], PO3862), pTX14-1 (*Bacillus thuringiensis subsp. israelensis* [65% similarity], P71099) (Andrup *et al.,* 2003), pTEF1 (*Enterococcus faecalis* [61% similarity], Q82YU6) and pER371 (*Streptococcus thermophillus* ST371 [74% similarity], O30928) (Solamain and Somkuti, 1998). Sequence analysis and visual inspection indicates that ORF4 encoded polypeptide contains three conserved motifs located in the N-terminal moiety (Fig. 4.7): (i) motif I (HNNR), of unknown function; (ii) motif II (NIY), which contains the putative catalytic tyrosine; and (iii) motif III (HNDEVSPHLH), also known as the 3H motif, probably involved in metal ion co-ordination (Garcillan-Barcia et al., 2009; Francia et al., 2004).The ORF5 encoded polypeptide contains three sequence motifs (N - 18 - FMTLATPN - 46 - NPHFHVLWR - 59 - AKYSAKDFE - 92 - C) (Fig. 4.8), similar to the Rep protein of pC194 group plasmids (Ilyina and Koonin, 1992).

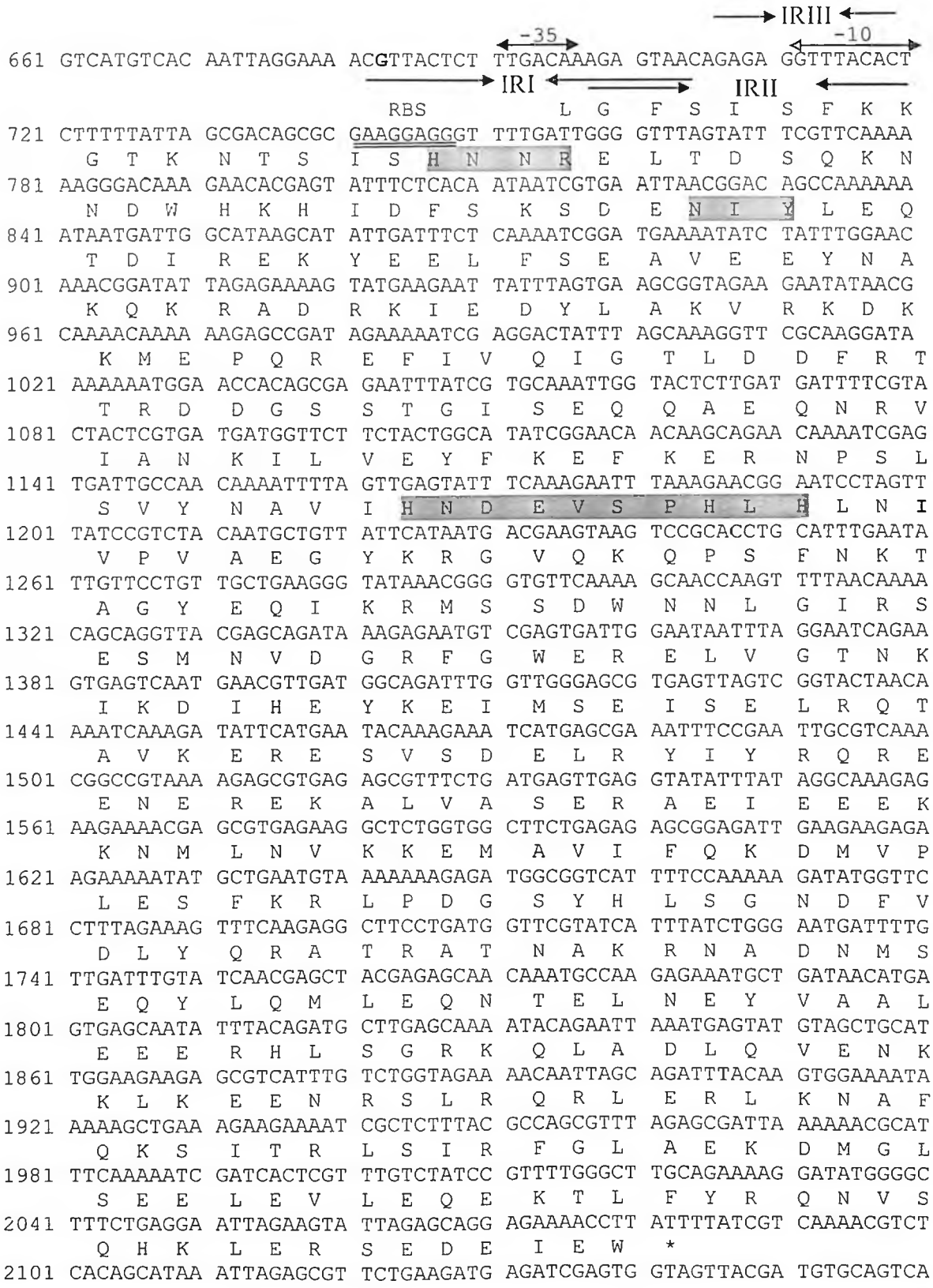


Fig. 4.7: Detailed DNA sequence of predicted *mob* gene and their upstream untranslated region, which includes origin of transfer: Amino acids deduced from the nucleotide sequence are specified using the standard one letter abbreviations. Conserved -35 and -10 regions of putative promoters are indicated as such are shown by double arrow head. Potential Shine-Dalgarno sequence (RBS) is double underlined. Inverted repeats (IR I , IR II & IR III) characterizing the putative *ori*T is indicated by arrow. Conserved sequence motifs, motif1, motif2 and motif3 as described by Garcillan-Barcia et al. (2009) are highlighted with dark grey.

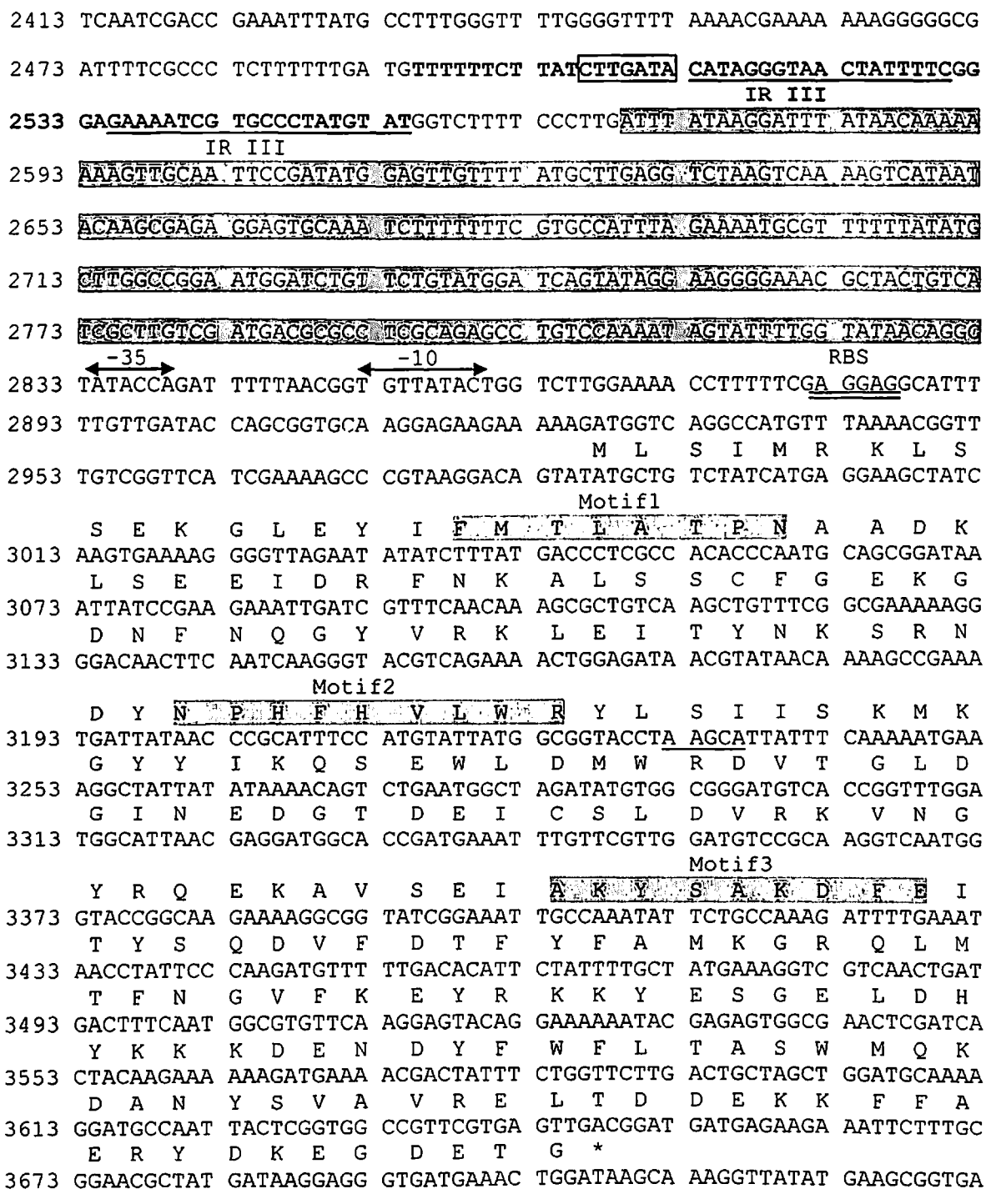


Fig. 4.8: Detailed DNA sequence of predicted *rep* gene and their upstream untranslated region, which includes *ori* and *ssoU* (*ssoA* is not shown): Amino acids deduced from the nucleotide sequence are specified using the standard one letter abbreviations. Conserved -35 and -10 regions of putative promoters are indicated as such are shown by double arrow head. Potential Shine-Dalgarno sequence (RBS) is double underlined. Inverted repeat (IR III) characterizing the *dso* (bold fece font sequence) is indicated by underlined sequence. Boxed bold face font sequence represents nic site of *dso*. Conserved motif 1, motif2 and motif3 amino acid sequences as described by Ilyina and Koonin (1992) are highlighted with light gray. Single strand origin *ssoU* sequence is highlighted with dark grey.

4.2.6. Identification of double-strand origin, single-strand origin & origin of trnsfer

Examination of DNA sequence in the region upstream from ORF5 revealed the presence of a 56-bp sequence, 5′-TTTTTTCTTATCTTGATACATAGGGTAACTA TTTTCGGGAGA AAATCGTGCCCTAT -3′ (located between nt 2495 and 2550 and with a free energy of -8.76 kcal/mol), that is homologous to the known double strand origin of plasmid pC194 (Fig. 4.9, PanelA). This homologue sequence contains a conserved nic site (CTTGATA) located in the loop of its secondary structure (Fig.4.9, Panel B). This region was exposed to its cognate replication protein for specific site recognition, nicking creation and initiation of the leading strand replication.

A

```
pNJAKD_dso          TTTTTTCTTATCTTGATACATAGG-GTAACTATTTTCGGGAGAA
pC194_dso           TTCTTTCTTATCTTGATA-ATAAGOGTAACTATTGCCGGC-GAG
Clustal Consensus   ** *************** *** * *********  ***  **
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B

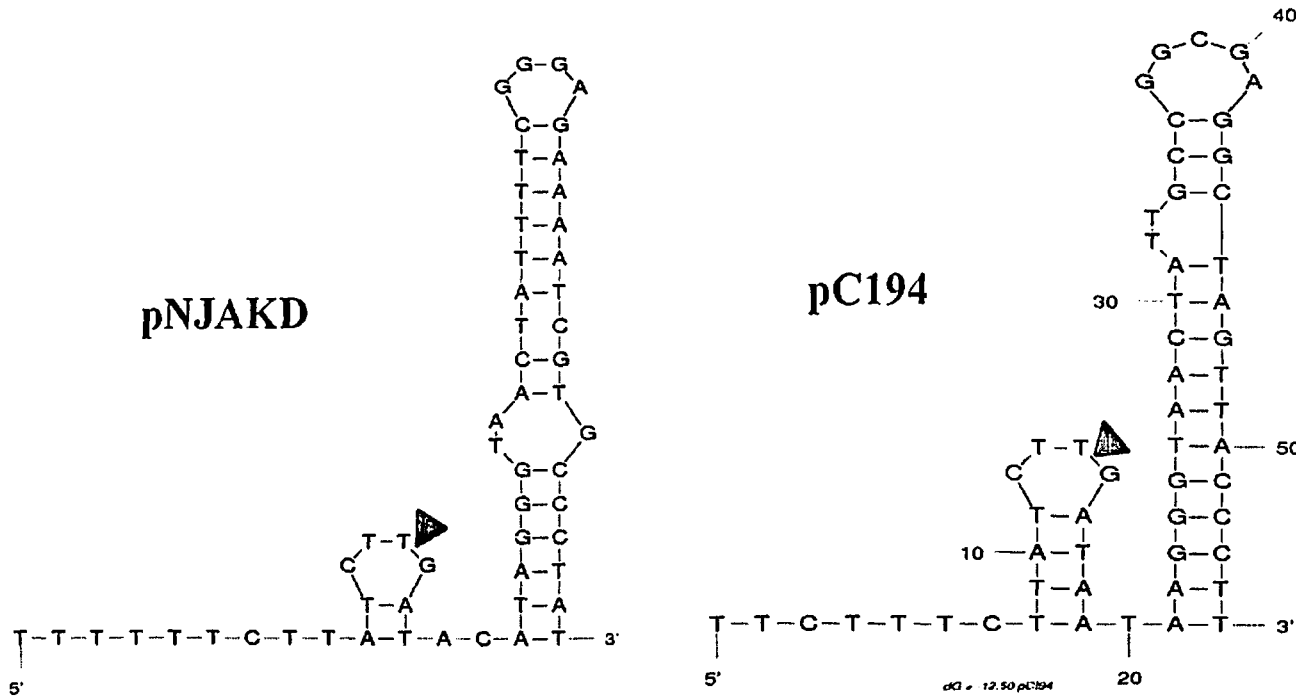
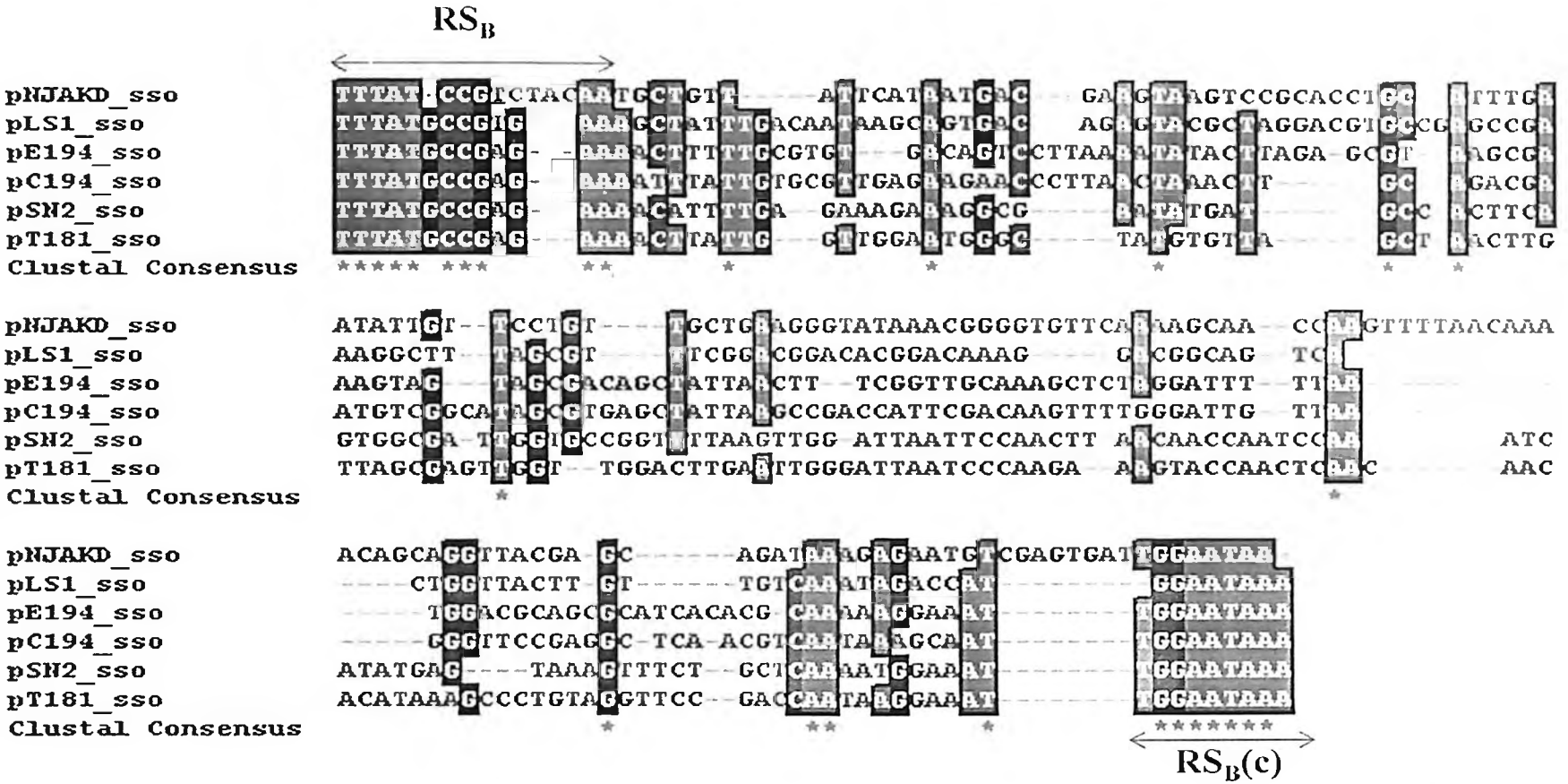


Fig. 4.9: Sequence and secondary structure based comparison of the pNJAKD *dso* with that of pC194:Panel A depicts the conserved sequences and the presence of nic- site of the *dso* of the pNJAKD plasmid; Asterics (*) symbol represents the conserved nucleotides. Sequence was aligned by using Clustal W multiple sequence alignment software. Panel B depicts the secondary structure comparison of *dso* of pNJAKD with pC194; ▲shape represents the *nic* site; Secondary structure was drawn by M. fold

Replication of the lagging strand of RCR plasmids initiates from single strand origins. Those origins usually have extensive secondary structures (Khan, 1997). Examination of pNJAKD sequence revealed the presence of two long palindromic sequences, which are able to generate long stem loop structures. A 168 bp long palindromic sequence, located in the coding sequence of ORF4 (between 1199 and 1366) showed significant level of similarity with *ssoA* group sequences and exhibiting a hairpin structure of calculated free energy -11.51 kcal/mol. Two regions RS_B and its partial complement RS_B(c) (Kramer *et al.*, 1997) were found to highly conserve among these s*soA*s (Fig. 4.10). A 265 bp long palindromic sequence located between 2569 and 2833 showed significant similarity to *ssoU* origin of pUB110 and pMV158 plasmids (Fig. 4.11) and produces a hairpin structure (3 perfect inverted repeats and 3 long imperfect inverted repeats) of calculated free energy -34.85 kcal/mol. The homologies around the RS_B conserved sequences and the presence of extensive secondary structures indicates the possibility of *ssoA* and *ssoU* type origin in the pNJAKD plasmid (Kramer *et al.*, 1999). An *oriT* is a short sequence of DNA that is necessary for transfer of a bacterial plasmid from a bacterial host to recipient during bacterial conjugation. A 40 bp long palindromic sequence located just upstream to ORF4 showed high degree of sequence and secondary structure similarity with *ori*T region of the pMV158 plasmid (Fig. 4.12) (Lorenzo-Diaz et al., 2011). The pNJAKD *ori*T consisted with three inverted repeats (IR1 , IR2 and IR3). Lorenzo-Diaz et al. (2011) has reported the same no. of inverted repeats in the *ori*T of pMV158. The IR1 of pNJAKD *ori*T showed similarity with IR1/3 of pMV158 *ori*T and could be the binding site for Mob protein.



4.10: Comparison of single strand origin of replication (*sso*) of pNJAKD with *ssoA* type sequences from plasmid pC194, pE194 and pSN2, pT181 and pLS1: The sequences were aligned using Clustal W multiple sequence alignment software. Double headed arrows epresent the conserved RS_B and RS_B(c) sequences. Figure prepared using BioEdit.

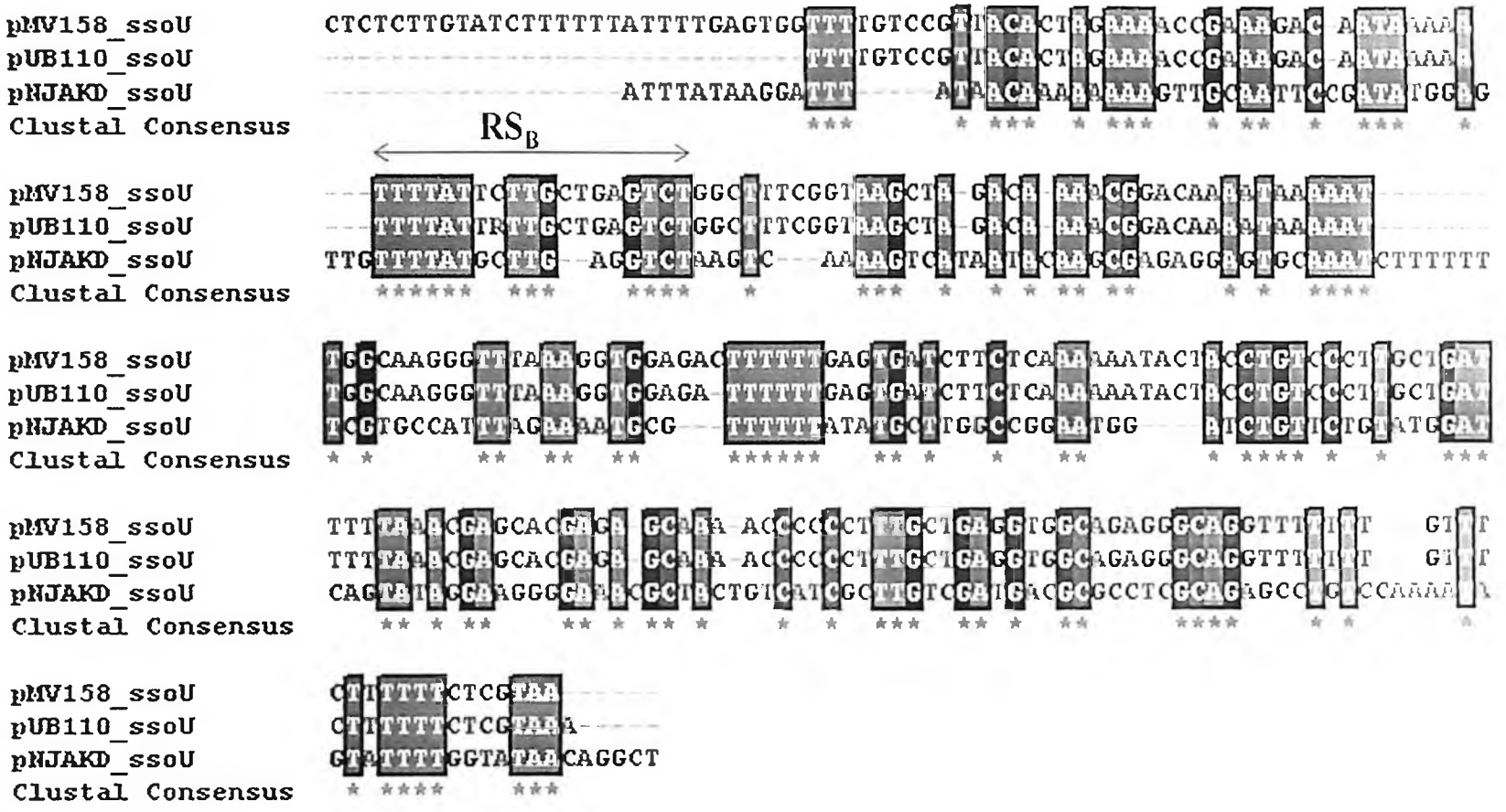


Fig. 4.11: Comparison of single strand origin of replication (*sso*) of pNJAKD with *ssoU* type sequences from plasmid pUB110 and pMV158: The sequences were aligned using Clustal W multiple sequence alignment software. Asterics (*) symbol represents the conserved amino acid sequence; double headed arrow represents the conserved RS_B sequence. Figure prepared using BioEdit.

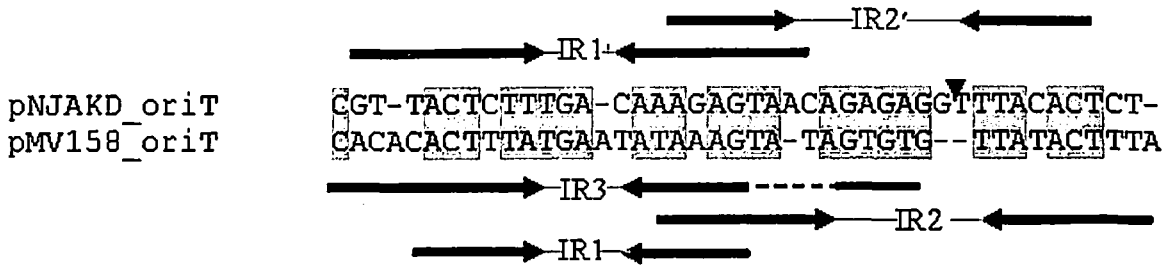


Fig. 4.12: Sequence and secondary structure based comparison of the pNJAKD *ori*T with that of pMV158: Conserved nucleotides are highlighted with light grey shade. Sequence was aligned by using Clustal W multiple sequence alignment software. IRI IRII and IRIII are three inverted repeates characterizing the putative *ori*T of pNJAKD plasmid. ▼ Symbol represents the nic site.

4.3. DISCUSSION

In the studies described here, the complete DNA sequence (3.825 kbp) of the natural plasmid pNJAKD was determined, structural and functional organization of this plasmid was deduced. The limited number of small size plasmids harbored by *E. faecium* were characterized, such as pMBB1 (2.85 kb, Wyckoff *et al.,* 1996), pRI1 (6.038 kb, Migura and Hasman, 2009). Sequence analysis of pNJAKD plasmid revealed the presence of five ORFs on the same strand and four regions which could form potential putative secondary structures. Two major ORFs, ORF4 and ORF5 were found to encode putative mobilization and replication proteins respectively.

Mobilization Module

The predicted mobilization protein starts with a rare TTG codon and shows a high degree of similarity with the mobilization or recombinase protein of the plasmids of a number of Gram-positive bacteria. The use of this alternative start codon has been reported in only a few exceptional cases (Gold, 1988). Interestingly, the regulatory virG gene of *Agrobacterium rhizogenes* (Aoyama *et al.,* 1989) and replication protein of pRS4 of *Pediococcus pentosaceus* (Alegre *et al.,* 2005) start with TTG. Perhaps this situation reflects a kind of translational control involved in the synthesis of optimal concentrations of this protein. Comparison of the predicted Mob protein of the Mob proteins of plasmids of the RC family of Gram-positive bacteria showed the presence of two conserved regions (RI and RII, Fig. 4.13).

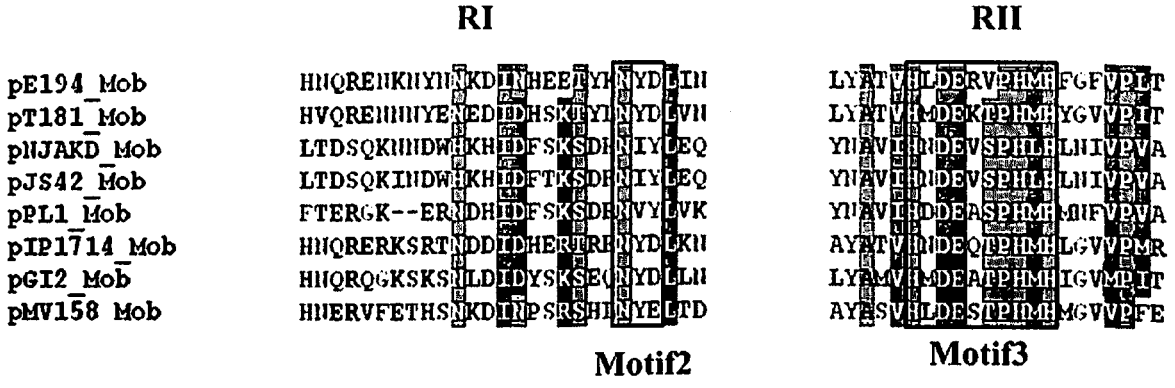


Fig. 4.13: Conserved regions in the Mob proteins of RC plasmids family: Clustal w multiple sequence alignment software were used for aligning the entire amino acid sequence of Mob protein of RCR plasmids. Two regions were found to be highly conserved region1 (RI) and region2 (RII). Conserved sequence motifs, motif2 and motif3 as described by Garcillan-Barcia et al. (2009) & Francia et al. (2004) are boxed. Figure prepared using BioEdit.

These regions are located at the N-terminal parts of the protein. RI contains the enzymatically active domain (Motif2: NXY) and RII contains putative metal binding domain (Motif3: HXDX2 [PU] HX) (Garcillan-Barcia et al., 2009; Francia et al., 2004). A structure based sequence alignment of 232 amino terminal residues (Mob_Pre domain, pfam: 01076) of predicted mobilization protein with relaxase domain of MobA (R1162, Monzingo *et al.*, 2007) is shown in Fig. 4.14. No significant sequence similarity was observed among them. However, they have both a catalytic tyrosine residue and an active site metal ion bound by three histidine residues. We have also observed that the amino terminal region of the predicted mobilization protein had a similar but not identical secondary structure with the relaxase domain of R1162 MobA (Fig. 4.14). A sequence and structure based alignment of predicted Mob proteins with others suggests the conservation of functional attributes of this protein. The DNA targets of the Mob protein is *ori*T. A putative *ori*T present in the pNJAKD plasmid is located just upstream to ORF4 and exhibits a high degree of similarity with *ori*T of pMV158 and pUB110 plasmids. The nick region of *ori*T of pMV158 was experimentally mapped by Guzman and Espinosa (1997). The *ori*T region of pNJAKD plasmid has been identified, based on similarities to previously described *ori*T regions, location (upstream from the mob-gene), sequence consensus, position of the conserved nic site and potential to form hairpin structures.

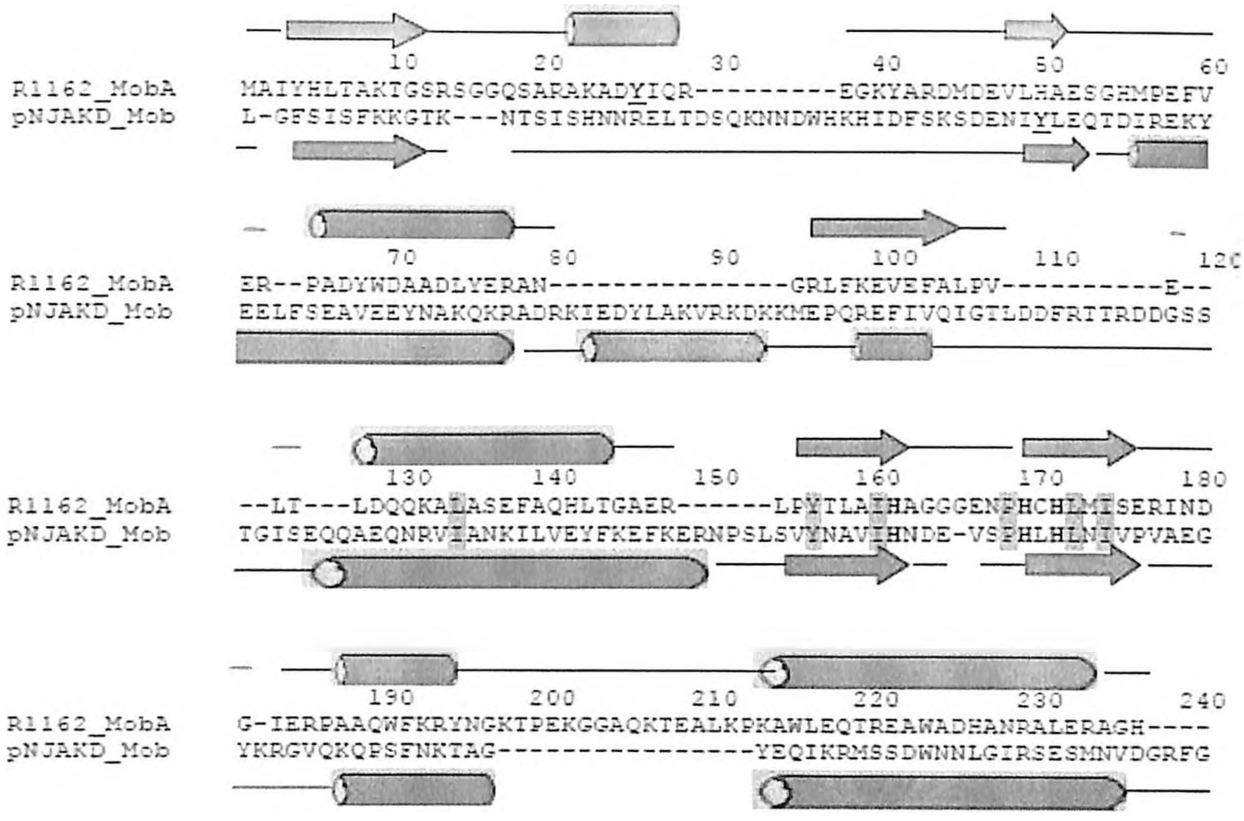


Fig. 4.14: The structure based sequence alignment of predicted N-terminal half of predicted pNJAKD Mob protein with relaxase domain of R1162 MobA:

The secondary structure elements of R1162 MobA and pNJAKD Mob are shown at the Top and at the bottom respectively. The conserved histidine residues are are shown as bold face font. The catalytic tyrosine residue is bold and underlined. The conserved hydrophobic residues are highlighted in light gray. Arrow and cylindrical shapes represent the β-sheets and α-helix respectively.

Replication Module

The replication module of pNJAKD plasmid consists of one double stranded origin, two single stranded origins and a replication protein. The replication protein exhibits similarity with those from a number of Gram-positive bacteria such as pC194 of *Staphylococcus aureus*, pTX14_1 of *Bacillus thuringiensis* subsp. Israelensis and pTEF1 of *Enterococcus faecalis*. Sequence alignment of pNJAKD encoded replication protein with the replication protein of pC194, pTX14_1 and pTEF1 showed the presence of three conserved sequence motifs (Fig. 4.8). Motif 2 (NPHFHVLWR) includes two histidine residues which could be involved in metal ion co-ordination. Motif3 (AKYSAKDFE) contains the tyrosine required to cleave one of the two DNA strands at the origin of dsDNA replication, generating 3′ - OH primer for DNA polymerase. A putative *dso* composed of 56 bp sequences is present, and is similar to the pC194 group plasmids. This is located upstream to the *rep* gene on the same strand. The identified nic site (CTTGATA) is located in the loop of the hairpin region as exemplified by plasmids of the pC194, pT181 and pE194 families (Gros *et. al.*, 1989). Based on the similarity of the replication protein and *dso* sequence, pNJAKD could be classified within the RCR plasmids of pC194 family (Khan, 2005). Based on sequence homologies, various types of *sso* have been described, including *ssoA,* which is present in plasmids pT181, pLS1, and pC194 (Novick, 1989), and *ssoU,* which is characteristic of plasmid pUB110 (Boe *et al.*, 1989). A more complex situation exists in the case of the pNJAKD plasmid, because it bears both types of *sso* (*ssoA* and *ssoU*) (predicted from sequence homology, hairpin structure and presence of RS_B site). This condition is rarely seen but has also been reported in the case of the pMV158 plasmid, which carries both *ssoA* and *ssoU* (Lorenz-Diaz and Espinosa 2009). The *ssoA* type origins appear to functions only in their natural hosts, while *ssoU* type origins are very efficient in various Gram-positive bacteria such as *Bacillus subtilis, Staphylococcus aureus, Streptococcus pneumonia* and *Lactococcus lactis* (Kramer *et al.*, 1999; Lorenz-Diaz and Espinosa, 2009). Thus, the presence of *ssoU* sequence in the pNJAKD plasmid may contribute to plasmid promiscuity. A southern hybridization experiment showed the positive signal for the presence of the pNJAKD plasmid single stranded DNA. Presence of pNJAKD plasmid single stranded DNA (Fig. 4.6) provides strong suggestive evidence that this plasmid replicates by the RCR mechanism (Khan, 2005).

Chapter 5

Promoter identification and characterization

Chapter 5

5.1. INTRODUCTION

The challenges associated with designing a new expression vector focuses around identifying a suitable novel promoter/ or origin of replication. Studies with putative promoter sequences present in LAB plasmids are limited. It is important to identify new promoters, from isolated LAB plasmids, which are simple to induce for genetic engineering applications. There have been reports on characterization of *Lactococcus lactis* lactose operon promoter (Van Rooijen *et al.,* 1992), xylose catabolic promoter for *Lactobacillus pentosus* (Lokman *et al.,* 1994), and acid inducible promoters (P1, P3, P170) of lactococcus lactis (Madsen *et. al.*, 2005), However, the toxicity of IPTG (Kosinski, 1992), the cost of IPTG and Xylose and the hazardous nature of acid has restricted the usage of these promoter systems especially in the industry (Li, 2007). So far, only the nisin inducible promoter system, named NICE, has found widespread use in LAB, particularly in *L. lactis* (Morelli et al., 2004). A problem associated with the nisin induction system, particularly in LAB other than *L.lactis*, is to find a correct expression level for *nis*R and *nis*K that can be tolerated by the host cell and still allow efficient induction. To the best of our knowledge few attempts have been made to use either plasmid borne or genomic DNA borne promoters isolated from LAB to produce recombinant gene product in *E. coli.*

In this chapter we report a strong temperature inducible promoter identified from the pNJAKD plasmid of *E. faecium*, which is functional in *E. coli* DH5α, JM101 and BL21 (DE3) at temperature change from 30 °C to either 37 °C or 42 °C.

5.2. RESULTS

5.2.1. Identification and analysis of promoter fragments

Promoter identification was done with the coding strand of the complete plasmid DNA as well as in the upstream regions of ORFs. We have found that ORF2, ORF4 and ORF5 contained promoters, but ORF1 and ORF3 didn't contain promoters. Promoters, their sequences and oligonucleotides from known TF binding sites are listed in Table 5.1. The nucleotide sequence analyses of putative promoter carrying fragments AKD1, AKD2, AKD3, AKD4, AKD5, AKD6 and AKD7 suggested that they all contained several potential promoter sequences exhibiting more than 60% homology with the prokaryotic -35 and -10 consensus hexamers, which are spaced by 14 to 23 bp nucleotides. Bioinformatic analysis of AKD1, AKD2, AKD4, AKD5, AKD6 and AKD7 promoters did not show the existence of promoter structure recognized by the sigma (σ) factor of Gram-negative and Gram-positive bacteria (Table 5.1).One is at -10 region and other is just upstream to -35 region (Fig. 5.1).

Table 5.1: Promoters, their sequences and oligonucleotides from Known TF binding sites

Sr. No.	Promoter	Position in pNJAKD plasmid	Promoter Sequences [Promoter Synthesis Strategy]	Length (bp)	Oligonucleotides from known TF binding sites
1	AKD1	3019-3127	cagctgAAAGGGGTTAGAATATATCTTTATGACCCTCGCCACACCCAATGCAGCGGATAAATTATCCGAAGAAAT TGATCGTTCAACAAAGCGCTGTGAAGCTGTTTCGGCGccatgg*catg* **[P]**	107	deoR: TTAGAATA
2	AKD2	3019-3247	CagctgAAAGGGGTTAGAATATATCTTTATGACCCTCGCCACACCCAATGCAGCGGATAAATTATCCGAAGAAAT TGATCGTTTCAACAAAGCGCTGTGAAGCTGTTTCGGCGAAAAAGGGGACAACTTCAATCAAGGGTAGGTCAGAAA ACTGGAGATAACGTATAACAAAAGCCGAAATGATTATAACCCGCATTTCACTTGATTATGGCGGTACCTAAGCAT TATTTCAAAAccatgg*catg* **[P]**	229	deoR: TTAGAATA
3	AKD3	2749-2895	cagctgTAGGAAGGGGAAACGCTACTGTCATCGCTTGTCGATGACGCGCCTCGCAGAGCCTGTCCAAAATAGTAT TTTGGTATAACAGGCTATACCAGATTTTTAACGGTGTTATACTGGTCTTGGAAAACCTTTTTCGAGGAGGCATT TTTGccatgg*catg* **[C]**	147	rpoD17: ATAACAGG rpoD17: GTTATACT
4	AKD4	1-171	cagctgCTGTGGAAAATGCTTATTTTCAAGTGTTCGGTGAACACCATAGCCCTGAATGGGATCGTGCTATTCTTG ATGATATGGCGGAAATGCACGAGAAATTGTGGAGGGCGCTAAATGACTAATCAAATTGGTGTTATTTATAGGAAT GAATCTAAGGGTAAGGAGTTGTAATTCccatg*catg* **[C]**	171	No site
5	AKD5	661-755	CagctgGTCATGTCACAATTAGGAAAACGTTACTCTTTGACAAAGAGTAACAGAGAGGTTTACACTCTTTTTATT AGCGACAGCGCGAAGGAGGGTTTTGAccatgg*catg* **[C]**	95	crp: TCACAATT trpR: TACTCTTT argR2: TTTTTATT
6	AKD6	2699-2750	GCGTTTTTTATATGCTTGGCGGGAATGGATCTGTTCTGTATGTTAGGAGTATAccatg*catg* **[C]**	53	No site
7	AKD7	2495-2535	TTTTTTCTTATCTTGATACATAGGGTAACTATTTTCGGGAGCCGCAGGATTAccatg*catg* **[C]**	52	No site

The *cis*-acting element -35 and -10 are highlighted with dark grey and light grey respectively. P: PCR based synthesis; C: Chemical based synthesis as oligonucleotides. Small letters in the sequences represents restriction site

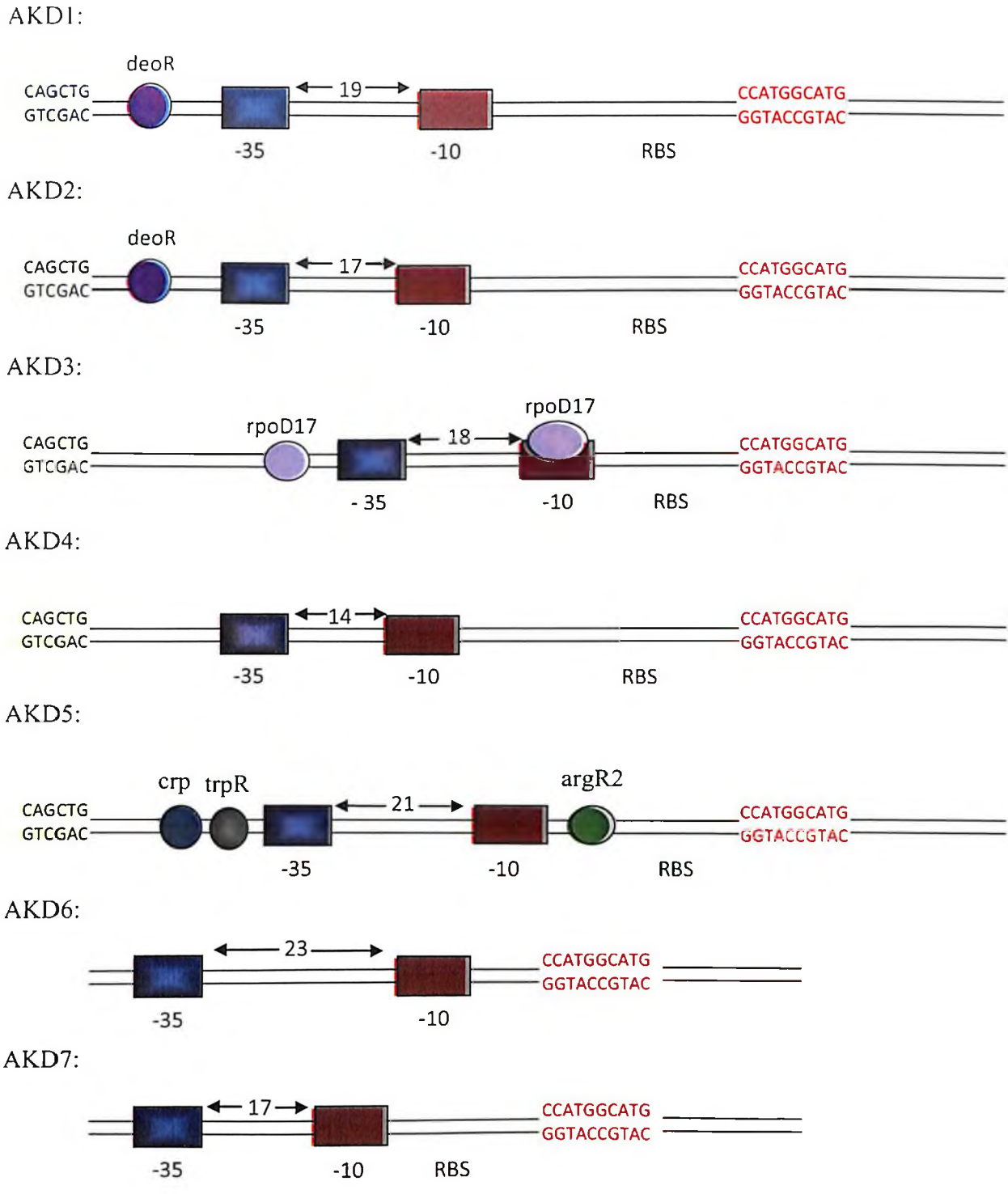


Fig. 5.1: Diagramatic representation of -35, -10 regions, spacing between -35 and -10 and *cis* site for known transcription binding sites; red font and green font letter represent *Nco* I and *Pvu* II restriction sites respectively

5.2.2. Promoter strength determination

The strength of the seven putative promoters (AKD1, AKD2, AKD3, AKD4, AKD5, AKD6 and AKD7) were determined by measuring the expression level of eGFP in the various strains of *E. coli* (JM101, DH5α, BL21(DE3)) at different temperature (30 °C, 37 °C and 42 °C) and time periods (4 h, 6 h, 8 h and 12 h) (Table 5.2, Table 5.3, and Table 5.4).

Significant expression profile was observed only by AKD3 promoters in *E. coli* strains as compared to other promoters (Table 5.2, Table 5.3, and Table 5.4).

In *E. coli* JM101 at 37 °C, the expression was high at 12 hours [1984.93 ± 22.65 (counts sec^{-1} ± SD) OD^{-1}] followed by 8 hours [1839.48 ± 33.78 (counts sec^{-1} ± SD) OD^{-1}] and then 6 hours [1683.88 ± 11.64 (counts sec^{-1} ± SD) OD^{-1}], and 4 hours [1053.37 ± 10.9 (counts sec^{-1} ± SD) OD^{-1}] (Table 5.2). In *E. coli* JM101 at 42 °C, the expression was high at 6 hours [7590.69 ± 42.90 (counts sec^{-1} ± SD) OD^{-1}] followed by 12 hours [6763.98 ± 6.20 (counts sec^{-1} ± SD) OD^{-1}] and then 8 hours [6678.62 ± 23.72 (counts sec^{-1} ± SD) OD^{-1}], and 4 hours [(3950.33 ± 11.19 (counts sec^{-1} ± SD) OD^{-1}] (Table 5.2). In *E. coli* DH5α at 37 °C, the expression was high at 12 hours [802.58 ± 9.11 (counts sec^{-1} ± SD) OD^{-1}] followed by 8 hours [901.03 ± 7.91(counts sec^{-1} ± SD) OD^{-1}] and then 6 hours [611.14 ± 5.53 (counts sec^{-1} ± SD) OD^{-1}], and 4 hours (554.77 ± 5.62) (Table 5.3).

In *E. coli* DH5α at 42 °C, the expression was high at 8 hours [6842.91 ± 33.04 (counts sec^{-1} ± SD) OD^{-1}] followed by 12 hours [4644.64 ± 17.83 (counts sec^{-1} ± SD) OD^{-1}] and then 6 hours [3214.07 ± 1.25 (counts sec^{-1} ± SD) OD^{-1}], and 4 hours [2328.05 ± 23.4 (counts sec^{-1} ± SD) OD^{-1}] (Table 5.3). In *E. coli* BL21(DE3) at 37 °C, the expression was high at 6 hours [8902.00 ± 97.71 (counts sec^{-1} ± SD) OD^{-1}] followed by 8 hours [7770.86 ± 11.61 (counts sec^{-1} ± SD) OD^{-1}] and then 12 hours [7498.92 ± 5.20 (counts sec^{-1} ± SD) OD^{-1}], and 4 hours [5489.04 ± 26.84 (counts sec^{-1} ± SD) OD^{-1}] (Table 5.4). In *E. coli* BL21(DE3) at 42 °C, the expression was high at 6 hours [7474.17 ± 67.26 (counts sec^{-1} ± SD) OD^{-1}] followed by 12 hours [7267.31 ± 16.39 (counts sec^{-1} ± SD) OD^{-1}] and then 8 hours [6703.40 ± 8.55 (counts sec^{-1} ± SD) OD^{-1}], and 4 hours [6571.26 ± 44.15 (counts sec^{-1} ± SD) OD^{-1}] (Table 5.4).

Table 5.2: Expression profile of eEGFP [(counts sec^{-1} ± SD) OD^{-1}][a] under control with *in-vitro* synthesized promoters in *E.coli* JM101

Promoter (IT→ / ITMP►)	4h			6h			8h			12h		
	30 °C	37 °C	42 °C	30 °C	37 °C	42 °C	30 °C	37 °C	42 °C	30 °C	37 °C	42 °C
AKD1	319.83 ± 4.7	276.38 ± 6.71	318.56 ± 7.51	284.85 ± 4.82	292.57 ± 9.78	340.90 ± 9.13	273.61 ± 7.14	307.74 ± 11.78	337.44 ± 4.80	287.28 ± 2.62	319.20 ± 7.57	328.35 ± 2.27
AKD2	308.73 ± 6.10	313.48 ± 2.86	293.41 ± 0.43	279.65 ± 7.18	308.65 ± 21.26	288.63 ± 2.21	269.77 ± 2.07	319.68 ± 7.37	274.83 ± 3.23	279.54 ± 7.50	330.28 ± 14.55	270.37 ± 3.61
AKD3	407.52 ±10.94	1053.37 ± 10.96	3950.33 ± 11.19	377.98 ± 0.26	1683.88 ± 11.64	7590.69 ± 42.90	364.99 ± 8.48	1839.48 ± 33.78	6678.62 ± 23.72	359.40 ± 8.74	1984.93 ± 22.65	6763.98 ± 6.20
AKD4	328.71 ± 1.21	316.5 ± 10.88	316.40 ± 4.59	299.93 ± 3.68	299.93 ± 10.34	303.24 ± 3.36	290.88 ± 1.00	249.09 ± 4.19	333.96 ± 9.68	314.44 ± 6.15	352.32 ± 13.58	379.43 ± 4.40
AKD5	315.53 ± 5.96	301.34 ± 3.41	299.99 ± 9.31	301.13 ± 4.36	304.43 ± 4.34	311.14 ± 6.92	299.18 ± 5.69	283.34 ± 3.43	304.43 ± 6.81	281.81 ± 8.32	301.10 ± 1.32	279.73 ± 7.69
AKD6	319.18 ± 9.13	311.12 ± 1.34	303.34 ± 6.32	307.69 ± 6.43	306.61 ± 3.25	299.13 ± 9.32	284.34 ± 6.31	281.19 ± 6.32	291.34 ± 3.42	279.34 ± 3.69	299.34 ± 6.31	287.37 ± 4.32
AKD7	314.45 ± 4.32	303.34 ± 6.32	306.31 ± 3.43	291.13 ± 9.34	284.45 ± 3.69	284.43 ± 4.43	280.19 ± 1.32	290.93 ± 1.43	315.32 ± 6.41	283.14 ± 3.63	271.17 ± 7.19	269.34 ± 6.91
Control												
LacP	355.29 ± 5.68	1411.65 ± 45.68	4607.25 ± 77.13	357.63 ± 2.62	1905.51 ± 13.89	5997.37 ± 44.90	360.05 ± 7.19	1976.65 ± 15.40	5835.09 ± 44.90	403.66 ± 4.22	2133.07 ± 20.90	6736.70 ± 46.96
Promoterless	278.89 ±11.51	254.43 ± 2.91	294.18 ± 7.16	230.93 ± 1.55	253.54 ± 0.98	284.29 ± 6.61	231.48 ± 1.90	245.92 ± 7.62	258.23 ± 13.80	238.34 ± 4.68	291.36 ± 5.17	318.47 ± 5.27
E.coli JM101	304.36 ± 4.14	278.80 ± 6.88	287.40 ± 6.45	263.45 ± 3.22	297.51 ± 7.78	270.64 ± 6.26	250.68 ± 9.80	266.80 ± 5.28	255.34 ± 9.54	276.42 ± 6.73	302.90 ± 4.46	290.12 ± 6.91

IT: induction time; ITMP: induction temperature; a: values are averages of three independent experiments in triplicate ± standard deviations.

Table 5.3: Expression profile of eGFP [(counts sec^{-1} ± SD) OD^{-1}][a] under control with *in-vitro* synthesized promoters in *E.coli* DH5α

Promoter (IT→ / ITMP▶)	4h			6h			8h			12h		
	30 °C	37 °C	42 °C	30 °C	37 °C	42 °C	30 °C	37 °C	42 °C	30 °C	37 °C	42 °C
AKD1	338.24 ± 5.25	286.80 ± 12.46	322.76 ± 6.80	277.88 ± 10.42	265.99 ± 2.24	283.06 ± 3.16	253.36 ± 7.40	288.65 ± 1.63	307.19 ± 7.84	266.45 ± 3.31	316.91 ± 3.33	337.08 ± 3.00
AKD2	327.80 ± 5.18	290.75 ± 7.63	299.59 ± 1.98	285.67 ± 0.36	270.42 ± 4.71	288.55 ± 6.62	264.20 ± 6.47	277.54 ± 3.78	287.37 ± 5.57	255.76 ± 4.00	312.37 ± 5.88	309.83 ± 7.97
AKD3	433.54 ± 13.57	554.77 ± 5.62	2328.05 ± 23.4	357.56 ± 4.74	611.14 ± 5.53	3214.07 ± 1.25	403.88 ± 10.56	901.03 ± 7.91	6842.91 ± 33.04	310.96 ± 14.50	802.58 ± 9.11	4644.64 ± 17.83
AKD4	317.80 ± 18.28	274.91 ± 9.99	293.52 ± 9.66	298.93 ± 14.3	299.34 ± 3.13	289.33 ±3.63	271.73 ± 3.09	293.83 ± 5.40	257.88 ± 3.05	284.55 ± 4.54	315.10 ± 8.61	290.24 ± 11.16
AKD5	311.46 ± 6.32	298.45 ± 8.32	301.43 ± 5.69	278.34 ± 3.33	283.67 ± 9.37	284.45 ± 6.32	264.65 ± 8.32	291.32 ± 3.43	263.34 ± 3.25	274.65 ± 6.89	299.32 ± 6.91	313.36 ± 3.45
AKD6	303.34 ± 1.48	301.69 ± 13.43	299.94 ± 5.67	303.63 ± 9.89	274.61 ± 6.36	291.33 ± 12.30	293.32 ± 6.45	283.46 ± 6.32	259.32 ± 6.33	291. 13 ± 3.40	301.32 ± 6.85	316.16 ± 6.37
AKD7	289.93 ± 10.12	295.98 ± 3.43	300.01 ± 1.33	302.24 ± 12.36	285.34 ± 6.54	298.32 ± 3.63	291.21 ± 9.31	286.64 ± 3.65	281.63 ± 3.69	299.18 ± 9.31	311.13 ± 3.43	331.31 ± 4.35
Control												
LacP	363.56 ± 8.53	552.88 ± 3.08	2839. 09 ± 18.22	363.67 ± 7.18	626.93 ± 5.01	4766.73 ± 19.01	339.69 ± 5.12	610.56 ± 10.27	5368.44 ± 27.46	360.86 ± 5.71	698.30 ± 2.22	6015.64 ± 78.42
Promoterless	268.80 ± 7.86	292.99 ± 9.83	287.76 ± 8.77	251.99 ± 4.68	278.13 ± 8.49	257.78 ± 2.64	255.76 ± 6.50	257.44 ± 3.15	245.59 ± 6.79	237.45 ± 6.55	293.99 ± 11.15	286.78 ± 8.52
E.coli DH5α	281.05 ± 4.54	291.94 ± 9.54	293.06 ± 7.09	247.47 ± 6.67	272.58 ± 2.30	291.72 ± 6.46	231.76 ± 12.20	257.92 ± 0.87	274.87 ± 3.14	239.25 ± 2.98	270.44 ± 4.45	292.15 ± 1.40

IT: induction time; ITMP: induction temperature; a: values are averages of three independent experiments in triplicate ± standard deviations.

Table 5.4: Expression profile of eGFP [(counts sec^{-1} ± SD) OD^{-1}][a] under control with *in-vitro* synthesized promoters in *E.coli* BL21(DE3)

Promoter (IT→ / ITMP→)	4h			6h			8h			12h		
	30 °C	37 °C	42 °C	30 °C	37 °C	42 °C	30 °C	37 °C	42 °C	30 °C	37 °C	42 °C
AKD1	330.32 ± 4.65	266.70 ± 6.54	275.27 ± 1.96	305.47 ± 2.03	285.24 ± 3.10	269.61 ± 0.43	315.61 ± 5.41	287.91 ± 5.90	256.57 ± 3.57	273.49 ± 1.55	321.22 ± 6.37	285.67 ± 1.05
AKD2	310.21 ± 3.57	278.01 ± 3.83	209.67 ± 1.19	283.79 ± 8.70	304.58 ± 11.75	276.09 ± 4.11	304.30 ± 3.43	309.39 ± 11.61	276.17 ± 2.16	268.44 ± 2.85	326.47 ± 6.54	294.00 ± 2.62
AKD3	1624.36 ± 16.70	5489.04 ± 26.84	6571.26 ± 44.15	2216.99 ± 9.55	8902.00 ± 97.71	7474.17 ± 67.26	2276.41 ± 7.70	7770.86 ± 11.61	6703.40 ± 8.55	2061.51 ± 33.38	7498.92 ± 5.20	7267.31 ± 16.39
AKD4	323.50 ± 17.19	282.96 ± 14.69	303.58 ± 4.43	291.13 ± 3.45	281.43 ± 9.31	266.34 ± 3.43	266.31 ± 2.21	270.97 ± 4.66	269.60 ± 3.39	245.62 ± 50.83	310.28 ± 10.00	298.58 ± 9.04
AKD5	281.34 ± 6.71	285.34 ± 6.32	281.01 ± 1.34	299.36 ± 9.54	264.69 ± 6.45	272.13 ± 1.35	301.34 ± 3.42	281.34 ± 6.92	265.45 ± 5.32	281.34 ± 3.69	305.41 ± 8.34	299.34 ± 3.91
AKD6	289.93 ± 3.89	279.32 ± 3.42	274.43 ± 2.31	301.43 ± 3.84	281.34 ± 2.11	281.32 ± 3.45	303.43 ± 9.13	289.45 ± 3.4	274.45 ± 4.32	274.51 ±13.12	315.32 ± 3.45	288.43 ± 6.90
AKD7	279.13 ± 0.65	271.63 ± 3.45	264.69 ± 6.93	299.13 ± 6.13	283.13 ± 1.43	284.35 ± 6.92	299.93 ± 1.45	299.33 ± 6.93	281.13 ± 9.32	281.19 ± 3.43	301.34 ± 4.63	299.11 ± 5.32
Control												
LacP	2204.14 ± 39.61	7735.69 ± 26.81	4188.95 ± 66.72	2207.43 ± 6.77	7089.43 ± 75.35	6798.49 ± 30.76	1864.03 ± 29.33	6515.21 ± 54.25	6189.07 ± 24.34	1656.55 ± 18.54	6927.76 ± 75.48	7610.38 ± 79.47
Promoterless	278.59 ± 9.93	281.94 ± 1.55	289.14 ± 2.47	264.16 ± 10.23	286.58 ± 6.30	281.65 ± 9.15	242.86 ± 6.61	283.32 ± 4.04	254.30 ± 5.94	266.33 ± 9.60	289.79 ± 9.14	319.54 ± 3.60
E.coli BL21(DE3)	327.72 ± 4.07	313.67 ± 4.92	295.97 ± 10.35	298.67 ± 2.10	291.14 ± 3.85	293.60 ± 2.44	261.98 ± 9.70	289.61 ± 8.24	271.34 ± 6.37	282.88 ± 9.56	315.91 ± 8.46	321.94 ± 9.64

IT: induction time; ITMP: induction temperature; a: values are averages of three independent experiments in triplicate ± standard deviations.

The expression level of eGFP by AKD3 promoter was also compared at temperature change from 30 °C to 37 °C and 30 °C to 42 °C for 4 h, 6 h, 8 h and 12 h inductions (Table 5.5, Table 5.6). The magnitude of the induction levels upon temperature change varied from 1.3 to 20 fold, depending upon degree of change, time of induction and type of strains.

Highest expression was observed in JM101 (20 fold) at 6 h induction at temperature change from 30 °C to 42 °C (Fig 5.2, Table 5.6) and in DH5α (17 fold) at 8 h induction at temperature change from 30 °C to 42 °C (Fig. 5.3, Table 5.6). The AKD3 promoter also showed the significant 5.5 fold induction in JM101 at temperature change from 30 °C to 37 °C (Fig 5.2, Table 5.5). No significant induction by AKD3 promoter was observed in DH5α at temperature change from 30 °C to 37 °C (Fig. 5.3, Table 5.5). In BL21(DE3), the AKD3 promoter showed significant 4 fold induction at temperature change from 30 °C to 37 °C (Fig. 5.4, Table 5.5) and 30 °C to 42 °C (Fig. 5.4, Table 5.6) at 6 hours and 4 hours respectively.

Table 5.5: Level of eGFP expression by AKD3 promoter in *E. coli* at temperature changes from 30 °C to 37 °C

Strain	Time (h)	eGFP expression [(counts sec^{-1} ± SD) OD^{-1}][a]		Fold induction[b]
		30 °C	37 °C	
JM101	4	407.52 ±10.94	1053.37 ± 10.96	2.6
	6	377.98 ± 0.26	1683.88 ± 11.64	4.5
	8	364.99 ± 8.48	1839.48 ± 33.78	5
	12	359.40 ± 8.74	1984.93 ± 22.65	5.5
DH5α	4	433.54 ± 13.57	554.77 ± 5.62	1.3
	6	357.56 ± 4.74	611.14 ± 5.53	1.7
	8	403.88 ± 10.56	901.03 ± 7.91	2.2
	12	310.96 ± 14.50	802.58 ± 9.11	2.6
BL21(DE3)	4	1624.36 ± 16.70	5489.04 ± 26.84	3.4
	6	2216.99 ± 9.55	8902.00 ± 97.71	4
	8	2276.41 ± 7.70	7770.86 ± 11.61	3.4
	12	2061.51 ± 33.38	7498.92 ± 5.20	3.6

[a] values are averages of three independent experiments in triplicate ± standard deviations (SD); [b] Fold induction with respect to value of eGFP expression at 30 °C

Table 5.6: Level of eGFP expression by AKD3 promoter in *E. coli* at temperature changes from 30 °C to 42 °C

Strain	Time (h)	eGFP expression [(counts sec^{-1} ± SD) OD^{-1}][a]		Fold induction[b]
		30 °C	42 °C	
JM101	4	407.52 ±10.94	3950.33 ± 11.19	9.7
	6	377.98 ± 0.26	7590.69 ± 42.90	20
	8	364.99 ± 8.48	6678.62 ± 23.72	18.3
	12	359.40 ± 8.74	6763.98 ± 6.20	19
DH5α	4	433.54 ± 13.57	2328.05 ± 23.4	5.4
	6	357.56 ± 4.74	3214.07 ± 1.25	9
	8	403.88 ± 10.56	6842.91 ± 33.04	17
	12	310.96 ± 14.50	4644.64 ± 17.83	15
BL21(DE3)	4	1624.36 ± 16.70	6571.26 ± 44.15	4
	6	2216.99 ± 9.55	7474.17 ± 67.26	3.4
	8	2276.41 ± 7.70	6703.40 ± 8.55	3
	12	2061.51 ± 33.38	7267.31 ± 16.39	3.5

[a] values are averages of three independent experiments in triplicate ± standard deviations (SD); [b] Fold induction with respect to value of eGFP expression at 30 °C

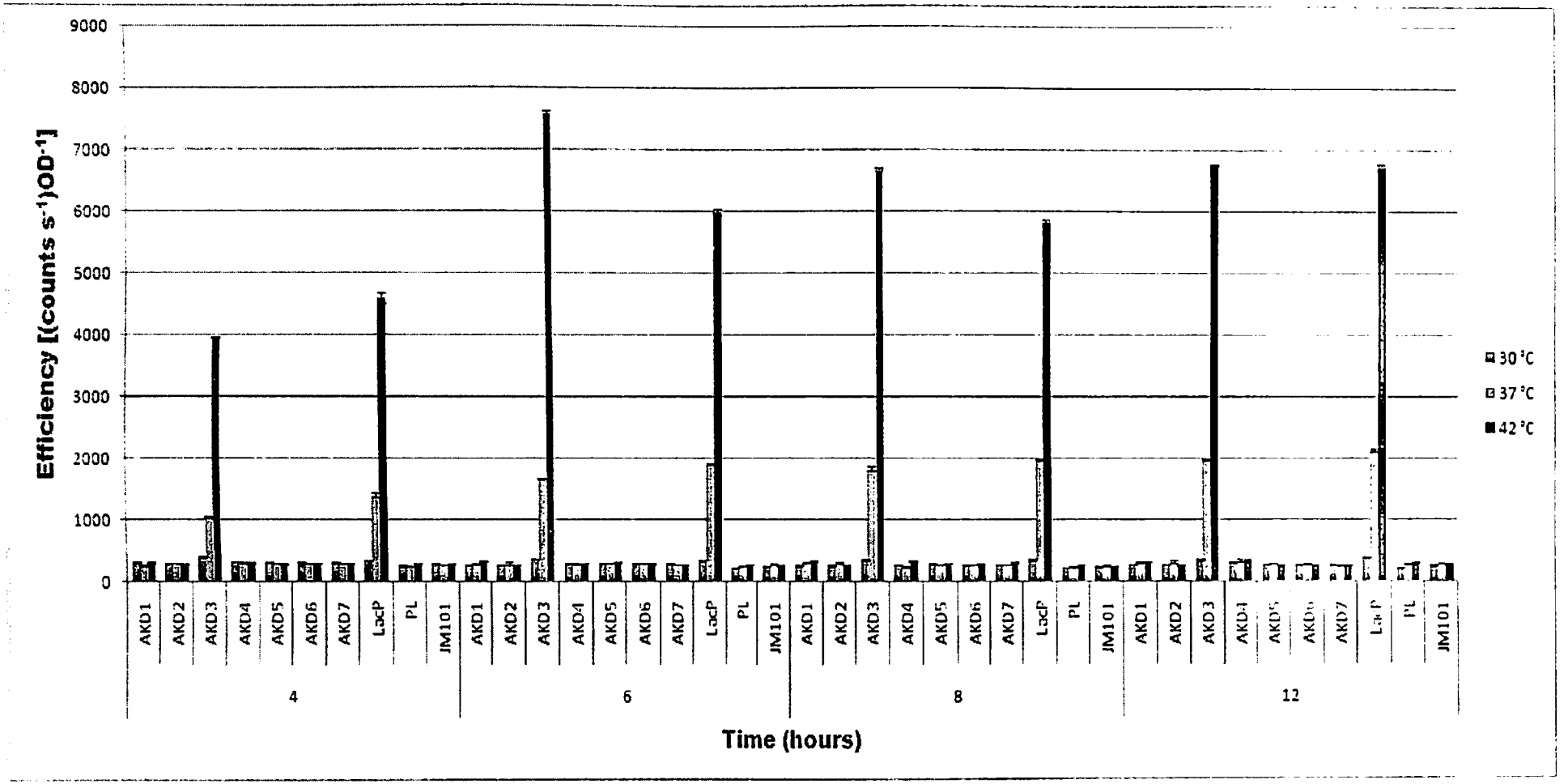


Fig. 5.2: Comparison of the expressed GFP intensities by *in-vitro* synthesized putative promoters (AKD1 to AKD7) in *E. coli* JM101 at temperature changes from either 30 °C to 37 °C or 30 °C to 42 °C. PL: promoterless pEGFP vector; Values are mean standard deviations of three independent experiments in triplicate.

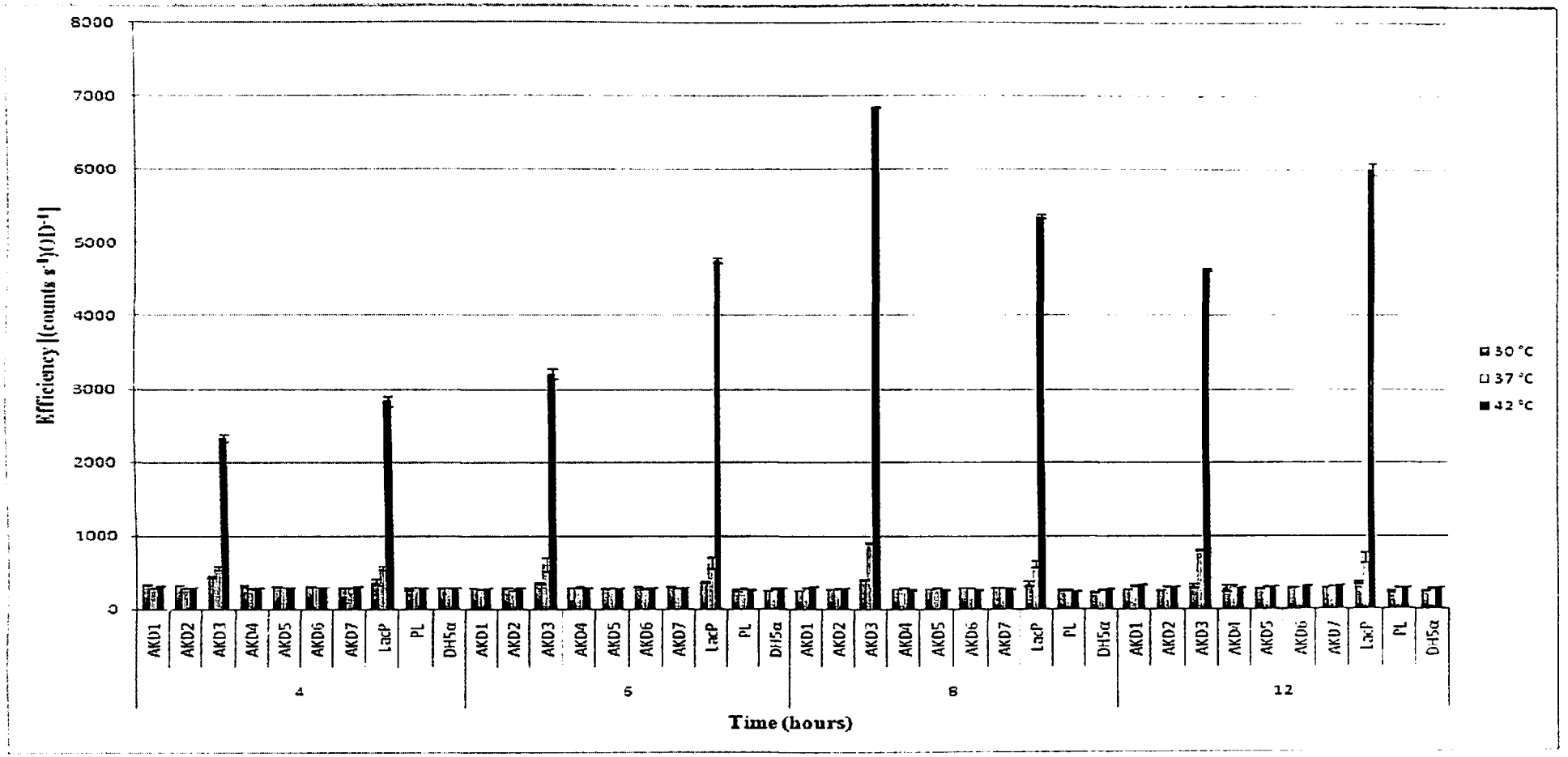


Fig. 5.3: Comparison of the expressed GFP intensities by *in-vitro* synthesized putative promoters (AKD1 to AKD7) in *E. coli* DH5α at temperature changes from either 30 °C to 37 °C or 30 °C to 42 °C. PL: promoterless pEGFP vector; Values are mean standard deviations of three independent experiments in triplicate.

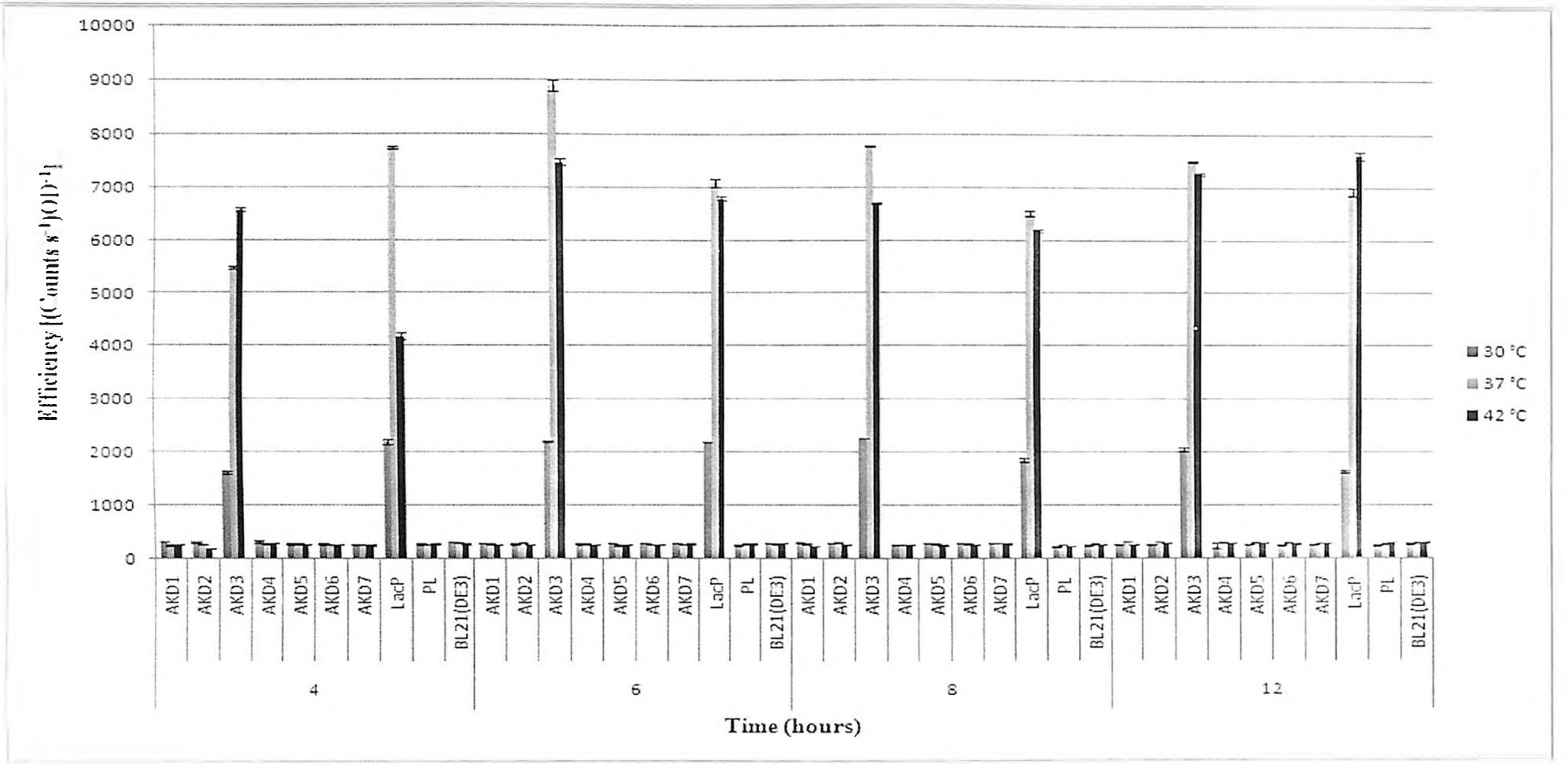


Fig. 5.4: Comparison of the expressed GFP intensities by *in-vitro* synthesized putative promoters (AKD1 to AKD7) in *E. coli* BL21(DE3) at temperature changes from either 30 °C to 37 °C or 30 °C to 42 °C. PL: promoterless pEGFP vector; Values are mean standard deviations of three independent experiments in triplicate.

To confirm the activity of promoters for eGFP expression, we initiated a reinvestigation of both promoter segments as present after the cloning experiment and also correlation of the activity based on the sequence analysis and, realized that due to the strategy adopted for the cloning of the synthesized promoter like region using the restriction enzyme (*Nco* I), two nucleotides were added at the 3 end of the promoter like region representing AKD1, AKD2, AKD3, AKD4, AKD5, AKD6 & AKD7. We have explained the promoter activity of AKD3 in the *E. coli* system and also the probable reasons for the lack of appreciable promoter activity for AKD1, AKD2, AKD4, AKD5, AKD6 &AKD7 in *E. coli* based system in the following paragraphs.

Transcription initiation control:

The transcriptional initiation control regions are given in table no. 5.7. Out of the seven identified promoters, only AKD3 has a well conserved -10 sequence (1 mismatch from consensus sequence). The AKD1, AKD4, AKD5, AKD6 and AKD7 are not well conserved -10 sequence (2 mismatch from consensus sequence). Similarly, the -10 region of AKD2 has 3 mismatche from the consensus sequence. The –16 region TG motif is often found 1 bp upstream of the –10 sequence in a large portion of Gram-positive bacterial promoters (Voskuil and Chambliss, 1998) and it enhances a step of transcription initiation which is typically performed by the -35 region. It is also known, that, the TG motif along with the -10 region is the minimum sequence necessary for promoter function in *E. coli.* The TG motif was observed in AKD3, AKD4 and AKD6 promoters, whereas, it is absent in AKD1, AKD2, AKD5 and AKD7. The UP element, a component of bacterial promoters located upstream of the -35 hexamer, increases transcription by interacting with the RNA polymerase α-subunit (Estrem et al., 1998). The consensus sequence for UP element is -59 nnAAA(A/T)(A/T)T (A/T)TTTT nn AAAAnnn-38 (Estrem et al., 1998) and it is highly conserved in AKD3. It is clear from this that, only AKD3 has highly conserved Gram-ve prokaryotic transcriptional control regions. Therefore, this is the probable reason that the promoter can show efficient expression. We have also predicted the effect of translational initiation control region for these promoters.

Table 5.7: Comparison of *in-vitro* synthesized promoters with the consensus prokaryotic elements

Promoter	-59/-39 AT- rich	-35 TTGACA	-16 TG	-10 TATAAT
AKD1	GTTAGAATATATCTTTATGACCC	TCGCCA	CACCCAATGCAGCGGA	TAAATT
AKD2	GATAACGTATAACAAAAGCCGAA	ATGATT	ATAACCCGCAT	TTCACT
AKD3	CAAAATAGTATTTTGGTATAACAGGCT	ATACCA	GATTTTTAACGGTGT	TATACT
AKD4	CGAGAAATTGTGGAGGGCGCTAA	ATGACT	AATCAAATTGG	TGTTAT
AKD5	CACAATTAGGAAAACGTTACTCT	TTGACA	AAGAGTAACAGAGAGGTT	TACACT
AKD6	GCGTTTTTTATATGC	TTGGCC	GGAATGGATCTGTTCTG	TATGTT
AKD7	TTTTTTCTTATC	TTGATA	CATAGGGTAAC	TATTTT

The TG motif is highlighted with dark grey

Translational initiation control:

The translational initiation control region extends from the Ribosome binding site (RBS) to the AUG start codon. Freier et al. (1986) have shown that efficient translation requires an easily accessible AUG start codon residing in a single stranded rather than a double stranded region of the hairpin loop of the translational initiation region (TIR). The secondary structure of TIR of *in-vitro* synthesized promoter fragments (except AKD6, because of the absence of Shine-Dalgarnov sequence in AKD6) were predicted by Mfold. The predicted secondary structure (Fig. 5.5) showed that AKD1, AKD4, AKD5 and AKD7 are present in double stranded regions of hairpin loops, whereas, AKD2 is in the single stranded loop region of the hairpin. AKD3 is in the single stranded region, not included in the hairpin structure.

de Smit & vanDuin (1990) showed that the expression is decreased in *E. coli* as the stability of the secondary structure of RBS is increased. To approach this apparent relationship in a more quantitative manner, the stability (ΔG_D: Loop dissocation free energy) of the translational initiator hairpin structures of the *in-vitro* synthesized promoter fragments were calculated by using the parameters of Friers et al. (1989). The ΔG_D values for TIR loop of synthesized promoters are given in table no. 5.8. It is clear from the table 5.8, that the stability of the TIR loop of AKD1, AKD2 and AKD5 are high, whereas, it is less for AKD3, AKD4 and AKD7.

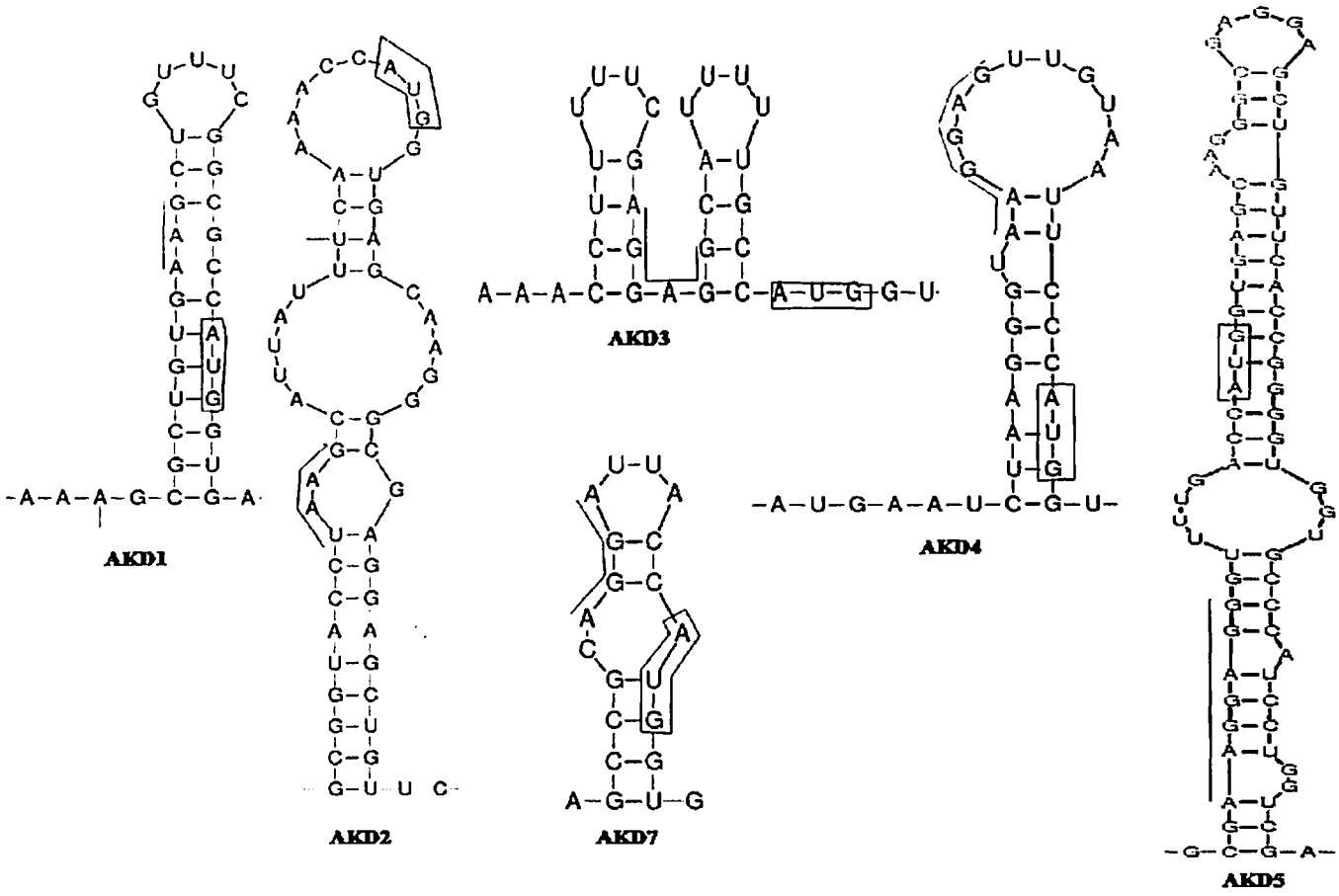


Fig. 5.5: Hairpin structure of translational initiator regions of *in-vitro* synthesized promoter fragments (CC included at 3′ end, just before ATG): Hairpin structure was predicted by Mfold software.

Table 5.8: Promoters (with CC), TIR sequence and their loop dissociation energy

Promoter	Distance S/D-ATG (nucleotides)	Sequence of TIR	ΔG_D for TIR secondary structure
AKD1	13	AAGCTGTTTCGGCGCCATG	-14.41
AKD2	15	TAAGCATTATTTCAAAACCATG	-17.52
AKD3	10	AGGAGGCATTTTTGCCATG	-8.32
AKD4	11	AAGGAGTTGTAATTCCCATG	-5.57
AKD5	10	AAGGAGGGTTTTGACCATG	-35.77
AKD7	5	AGGATTACCATG	-6.85

TIR: Translational initiation region; S/D-ATG: distance between Shine-Dalgarnov region and ATG start codon; ΔG_D: Loop dissociation energy at 25 °C; RBS and start ATG codon are underlined.

We also investigated the effect of additional CC bases (due to *Nco* I) included at 3 end of *in-vitro* synthesized promoter fragments. This affects the orientation of the AUG start codon and the stability of the predicted TIR loop for promoter fragments with CC (Fig.5.5) and without CC (Fig.5.6). Fig. 5.5 and Fig. 5.6 clearly indicates that, the orientation of the AUG starts are near about same for AKD1, AKD2, AKD4, AKD5 and AKD7 with and without CC, whereas, it is different for AKD3. In case of AKD3 without CC, the G and A bases of AUG start are paired, but with CC, the AUG start in the single stranded region, is not included in the loop

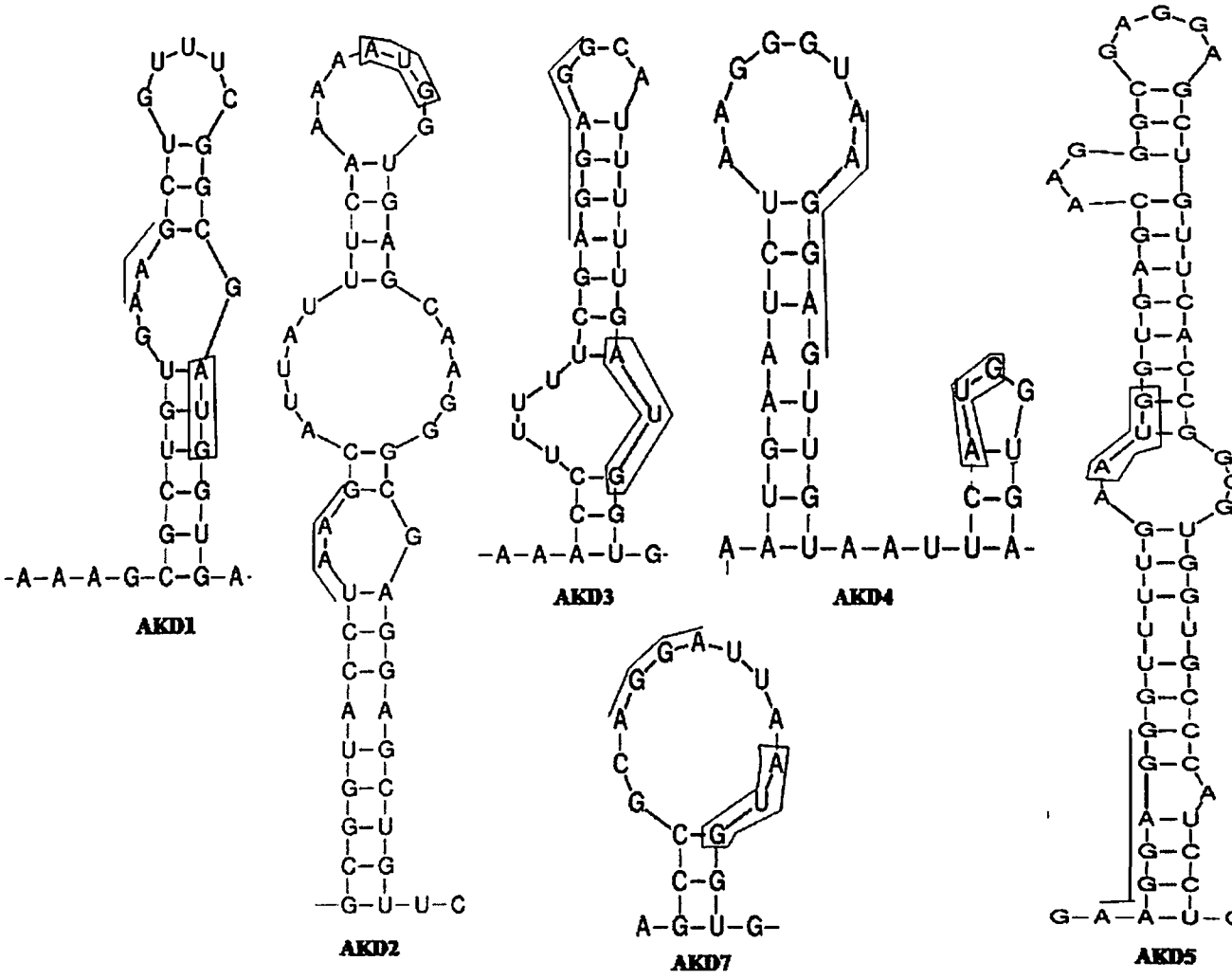


Fig. 5.6: Hairpin structure of translational initiator regions of *in-vitro* synthesized promoter fragments (CC is not included): Hairpin structure was predicted by Mfold software.

The dissociation energy of TIR loop of promoter fragments with CC and without CC are given in table no. 5.8 & 5.9. The dissociation energy of TIR loop of AKD1, AKD2, AKD4, AKD5 and AKD7 with CC are found to be higher than those without CC. Whereas, The dissociation energy of the TIR loop of AKD3 with CC is lesser than those without CC. We therefore conclude that the efficiency of the combined effect of the transcriptional initiation and translational initiation control regions for AKD3 promoter is high. This is the probable explanation for the AKD3 promoter showing good activity in *E. coli.* In contrast, for the other promoters, lack of appreciable activity seen is probably due to the presence of the CC bases.

Table 5.9: Promoters (without CC), TIR sequence and their loop dissociation energy

Promoter	Distance S/D-ATG (nucleotides)	Sequence of TIR	ΔG_D for TIR secondary structure
AKD1	11	AAGCTGTTTCGGCGATG	10
AKD2	13	TAAGCATTATTTCAAAAATG	-13.20
AKD3	8	AGGAGGCATTTTTGATG	-8.59
AKD4	9	AAGGAGTTGTAATTCATG	-4.46
AKD5	8	AAGGAGGGTTTTGAATG	-23.95
AKD7	3	AGGATTAATG	+1.3

TIR: Translational initiation region; S/D-ATG: distance between Shine-Dalgarnov region and ATG start codon; ΔG_D: Loop dissociation energy at 25 °C; RBS and start ATG codon are underlined.

5.3. DISCUSSION

There have been limited reports suggesting that promiscuous plasmids exist in the Gram +ve LAB which can also propagate in Gram –ve organisms (del Solar *et al.*, 1987, del Solar *et al.*, 1993). At, the time this work was initiated, we were interested in identifying promoters, which would be efficient in both Gram + ve, and Gram –ve organisms so as to enable creation of novel vectors with multiple use. Seven promoter sequences (AKD1, AKD2, AKD3, AKD4, AKD5, AKD6 and AKD7) from the pNJAKD plasmid of *Enterococcus faecium* were subsequently identified and evaluated the strength of each promoter in *E.coli* DH5α, JM101 and BL21(DE3). The AKD3 promoter showed moderate to highest activity at temperature change from 30 °C to either 37 °C or 42 °C. This is probably due to the greater affinity and occupancy of AKD3 promoter for RNA polymerase σ^{70} factor. Interestingly, the expression of eGFP under control of the AKD3 promoter in *E.coli* JM101 and DH5α was found to decrease after 6 hours and 8 hours respectively (at 42 °C). This could be explained by the fact that over expression of recombinant protein results in the activation of genes encoding chaperones and proteases (Valdez-Cruz et al., 2010). Chaperones such as DnaK repress the activity of σ^{70} (Valdez-Cruz et al., 2010) and proteases such as FtsH are involved in the degradation of unfolded proteins (Han and Lee, 2006), thereby controlling the expression of recombinant genes in *E.coli.*

At individual temperature such as 30 °C, 37 °C and 42 °C, the expression level of eGFP under control with AKD3 promoter was found to be much higher in BL21 (DE3) strain of *E.coli* in comparison to JM101 and DH5α. This is because of the absence of *lon* and *ompT* proteases in BL21 (DE3). Cells deficient in these proteases accumulate recombinant proteins at a high rate (Rozkov and Enfors, 2004). It was observed that the basal level of eGFP expression (expression of this gene at 30 °C) under control of AKD3 promoter in this strain is also high. This could be due to the presence of basal expression level of T7 polymerase in BL21 (DE3). The gene for T7 RNA polymerase under the control of the *lacUV5* promoter is present in this *E. coli* strain (Baneyx, 1999). This T7 polymerase strongly interacts with the T7 promoter. But it could weakly interact with the AKD3 promoter because of 60% similarity with the T7 promoter. Surprisingly, we found that the expression of eGFP gene under control with lac promoter (positive control) without IPTG in *E. coli* increases as

temperature increases from 30 °C to 37 °C and 42 °C. This is explained by bioinformatic analysis (Regulon DB) for regulators other than the commonly used regulators for lac promoter on the vector (pEGFP. The identified regulators and their binding sites are given in table 5.10.

Table 5.10: Regulatory site detected in pEGFP Vector

Regulator	Start	end	strand	score	sequence
LacI (E.coli)	179	199	+	27.12	AATTGTGAGCGGATAACAATT
ArcA	1400	1409	+	6.13	TGATAATAAT
	1465	1474	+	6.47	TGTTTATTTT
	2325	2334	+	6.25	AGTTATCTAC
	2467	2476	+	6.06	TTTTAATTTA
	2810	2819	+	6.32	TGTTACCAGT
	2873	2882	+	5.87	AGTTACCGGA
ArgR	2399	2412	+	8.57	TGATTAAGCATTGG
Crp	1058	1079	+	6.45	CAACTTGTCTGGTGTCAAAAAT
	1472	1493	+	6.07	TTTTCTAAATACATTCAAATAT
	1488	1509	+	6.39	AAATATGTATCCGCTCATGAGA
	1749	1770	+	7.15	AATGATGAGCACTTTTAAAGTT
	2449	2470	+	5.99	ATTGATTTAAAACTTCATTTTT
	3137	3158	+	6.34	TTTTGTGATGCTCGTCAGGGGG
CspA	1747	1751	+	10	CCAAT
CysB	2447	2477	+	9.89	AGATTGATTTAAAACTTCATTTTTAATTTAA
CytR	1485	1496	+	7.57	TTCAAATATGTA
	2119	2130	+	7.58	CGCAAACTATTA
DnaA	1471	1479	+	5.89	TTTTTCTAA
	1583	1591	+	6.57	TTATTCCCT
	1617	1625	+	6.32	TTTTGCTCA
	1789	1797	+	6	TTATCCCGT
	1836	1844	+	6.32	CTATTCTCA
	1912	1920	+	6.12	TTATGCAGT
	2000	2008	+	6.63	TTTTGCACA
	2327	2335	+	6.53	TTATCTACA
	2685	2693	+	6	TTTTCCGAA
	3222	3230	+	6.32	TTTTGCTCA
	3247	3255	+	6.66	TTATCCCCT
FadR	2230	2236	+	5.89	GGCTGGT
	3291	3297	+	5.93	AGCTGAT
FhlA	2536	2542	+	10.64	TTTTCGT
Fis	361	375	+	3.34	GGCCACAAGTTCAGC
Fnr	1467	1480	+	6.91	TTTATTTTTCTAAA
	2033	2046	+	7.16	TTGATCGTTGGGAA
	2409	2422	+	7.18	TTGGTAACTGTCAG
	2450	2463	+	7.31	TTGATTTAAAACTT
Fur			+		
GcvA	2803	2807	+	10	CTAAT

GlnG	57	62	+	5.01	TGGCAC
	154	159	+	4.96	TGCTTC
	323	328	+	5.14	TGGTGC
	2823	2828	+	5.09	TGCTGC
	3217	3222	+	4.85	TGGCCT
GlpR	1446	1465	+	6.29	TGTGCGCGGAACCCCTATTT
	2001	2020	+	5.82	TTTGCACAACATGGGGGATC
	2359	2378	+	5.83	GATGAACGAAATAGACAGAT
	2511	2530	+	5.87	CATGACCAAAATCCCTTAAC
	2601	2620	+	6.01	TCTGCGCGTAATCTGCTGCT
H-NS	106	116	+	6.07	TTAATGTGAGT
	126	132	+	6.21	CATTAGG
	146	156	+	7.28	ACACTTTATGC
	167	178	+	7.25	TATGTTGTGTGG
	180	187	+	6.91	ATTGTGAG
	194	204	+		ACAATTTCACA
	1477	1486	+	5.46	TAAATACATT
	1530	1539	+	5.23	TCAATAATAT
	1680	1689	+	5.02	GGGTTACATC
	2163	2172	+	5.07	ACAATTAATA
	2186	2195	+	5.54	CGGATAAAGT
	3090	3099	+	5.11	TCTTTATAGT
IHF	579	594	+	6.23	CACCATCTTCTTCAAG
	669	684	+	5.92	GGGCATCGACTTCAAG
	1475	1490	+	5.89	TCTAAATACATTCAAA
	1484	1499	+	6.15	ATTCAAATATGTATCC
	1528	1543	+	6.28	CTTCAATAATATTGAA
	1660	1675	+	5.89	GATCAGTTGGGTGCAC
	2118	2133	+	6.59	GCGCAAACTATTAACT
	2675	2690	+	6.41	TACCAACTCTTTTTCC
IciA	2244	2255	+	5.45	TGATAAATCTGG
	2399	2410	+	5.46	TGATTAAGCATT
	2502	2513	+	5.56	TGATAATCTCAT
	2835	2846	+	5.39	CGATAAGTCGTG
Lrp			+		
Lrp(SELEX)	1638	1662	-	11.54	ATCTTCAGCATCTTTTACTTTCACC
MalT	684	689	+	8.31	GGAGGA
	1975	1980	+	8.31	GGAGGA
	2184	2189	+	7.59	GGCGGA
	2358	2363	+	8.24	GGATGA
	3012	3017	+	7.59	GGCGGA
	3158	3163	+	7.59	GGCGGA
MetJ	738	746	+	6.97	CGTCTATAT
	1473	1481	+	6.67	TTTCTAAAT
	2328	2336	+	6.67	TATCTACAC
	2763	2771	+	6.73	CTTCAAGAA
MetR	1540	1546	+	9.57	TGAAAAA
	2980	2986	+	8.76	TGAGAAA

Mlc	101	106	+	5.98	CGCAAT
	1073	1078	+	5.35	CAAAAA
	1689	1694	+	5.99	CGAACT
	1734	1739	+	5.79	CGAAGA
	2136	2141	+	5.99	CGAACT
	2952	2957	+	5.99	CGAACT
NagC	1467	1482	+	14.80	TTTATTTTTCTAAATA
NagL			+		
OmpR (C box)	1523	1532	+	12.62	TGAAGCATTT
OmpR	1686	1652	+	7.88	TAAAAGA
	2456	2462	+	8.31	TAAAACT
	3171	3177	+	8.19	GAAAAAC
OxyR			+		
OxyR (SELEX)	1750	1795	-	13.66	ATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGG TATTATCCC
PdhR	1072	1077	+	6.20	TCAAAA
	1486	1491	+	6.35	TCAAAT
	1530	1535	+	6.85	TCAATA
	2167	2172	+	6.35	TTAATA
	2469	2474	+	6.51	TTAATT
RcsAB	1637	1644	+	5.06	TGGTGAAA
Sig70 (-10)			+		
YhiX	1402	1419	+	6.22	CTAAGAAACCATTATTAT

The regulator H-NS is known to control the gene expression with response to the temperature. The binding sites of H-NS in the vector are at -10, -35, -45, -68, +20, +1298, + 1351, + 1511, and + 2007 regions from the transcription start site of the lac-promoter. At 30 °C, binding of H-NS results in steric hindrance for RNA polymerase, but at 37 °, this steric hindrance might be released. Therefore, the expression of eGFP gene would increase as temperature increases from 30 °C to 37 °C. The expression of the eGFP gene under the control of the lac-promoter was also investigated in the presence of the IPTG at different temperature and time, but the expression of the eGFP was found to decrease after 4 hours, which could be due to degradation of the IPTG.

To conclude, we have reported a strong, temperature inducible AKD3 promoter, screened from the pNJAKD plasmid and functional in *E.coli* at temperature change from 30 °C to either 37 °C or 42 °C.

Chapter 6

CONCLUSIONS

Chapter 6

Summary

The major findings of the research are summarised as follows:

1. A total of 21 LAB isolates were isolated from milk, water, plant and soil samples, collected from different regions of india

2. Out of the 21 isolates, only 6 isolates harbor the plasmid(s). These isolates were identified at species level by 16s-rRNA gene amplification and sequencing. They are *E. faecium* DJ1, *E. faecium* DJ2, *E. faecium* DJ3, *S. epidermidis* NJ1, *W. confusa* NKD1 and *P. pentosaceus* NS01.

3. Plasmid of one isolate *E. faecium* DJ1 was characterized, and it was found that this plasmid is of small size (3.825 Kb) and replicates through RCR mechanism. This is proved by the detection of SS DNA through Southern blotting and hybridization experiments.

4. Sequence analysis of pNJAKD plamid revealed that, it contains five putative ORFs. No significant similarity was observed for the putative polypeptide encoded by ORF1. ORF2 and ORF3 encode a 13910 D and 9322 D peptides which shared homology with predicted protein CDS2 and CDS1 from *Enterococcus faecium* plasmid pRI1 respectively. There are two major ORFs, ORF4 & ORF5, which are similar to replication and mobilization proteins of number of Gram-positive bacteria.

5. Sequence analysis and visual inspection indicate that ORF4 encoded polypeptide contains three conserved motifs located in the N-terminal moiety (Fig. 5): (i) motif I (HNNR), of unknown function; (ii) motif II (NIY), which contains the putative catalytic tyrosine; and (iii) motif III (HNDEVSPHLH), also known as the 3H motif, probably involved in metal ion co-ordination (Garcillan-Barcia et al., 2009; Francia et al., 2004). The ORF5 encoded polypeptide contains three sequence motifs (N - 18 - FMTLATPN - 46 -

NPHFHVLWR - 59 - AKYSAKDFE - 92 - C) (Fig. 6), similar to the Rep protein of pC194 group plasmids (Ilyina and Koonin, 1992).

6. Sequence homology and Structural analysis identified a double-strand origin (similar to pC194), two single-stand origins (*ssoA* and *ssoU* type) and an origin of transfer (similar to pMV158) in the noncoding regions of pNJAKD plasmid.

7. Based on the similarity of the replication protein and *dso* sequence, pNJAKD could be classified with the RCR plasmids of pC194 family

8. The presence of RC replication module and mobilization region on pNJAKD plasmid indicates that this plasmid could possibly be transferred to a wider variety of hosts (i.e. broad host spectrum)

9. Seven promoter sequences (AKD1, AKD2, AKD3, AKD4, AKD5, AKD6 and AKD7) from the pNJAKD plasmid of *Enterococcus faecium* were identified and the strength of each promoter evaluated in *E.coli* DH5α, JM101 and BL21(DE3)

10. The AKD3 promoter showed moderate to highest activity at temperature shifts from 30 °C to either 37 °C or 42 °C

11. JM101 was found to be the most suitable strain for induction of eGFP under control of AKD3 promoter, because, there was 20 fold higher expression at temperature change from 30 °C to 42 °C at 6 hours induction

In conclusion, there are three major findings reported in this thesis:

Firstly, it has been identified a small size novel pNJAKD plasmid from natural isolates of *E.faecium* DJ1. Secondly, this pNJAKD plasmid was characterized at structural and functional level and found that this plasmid replicates through RCR mechanism. Finally, we have reported a strong, temperature inducible AKD3 promoter, screened from the pNJAKD plasmid and functional in *E.coli.*

Future perspectives

The promoters characterized in the present study can be utilized for the construction of LAB-*E. coli* shuttle expression vectors.

References

References

1. **Alegre, M. T., Rodriguez, M. C. and Mesas, J. M.** 2005. Nucleotide sequence, structural organization and host range of pRS4, a small cryptic Pediococcus pentosaceus plasmid that contains two cassettes commonly found in other lactic acid bacteria. *FEMS Microbiol. Lett.* **250:**151-156.

2. **Ali, A. A.** 2010. Beneficial Role of Lactic Acid Bacteria in Food Preservation and Human Health. *Res. J. Microbiol.* **5:**1213-1221.

3. **Altschul, S. F., Gish, W., Miller, W., Myers, E. W and Lipman, D. J.** 1990. Basic local alignment search tool. *J. Mol. Biol.* **215:**403-410.

4. **Anderson, D. G. and McKay, L.L.** 1983. A simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl. Environ. Microbiol.* **46:**549-552.

5. **Andrup, L., Jensen, G. B., Wilcks, A., Smidt, L., Hoflack, L. and Mahillon, J.** 2003. The patchwork nature of rolling-circle plasmids: comparison of six plasmids from two distinct *Bacillus thuringiensis* serotypes. *Plasmid* **49:**205–232.

6. **Arias, C. A. and Murray, B. E.** 2012. The rise of the *Enterococcus* beyond vancomycin resistance. *Nat. Rev.Microbiol.* **10:**266-278.

7. **Aoyama, T., Hirayama, T., Tamamoto, S. and Oka, A.** 1989. Putative start codon TTG for the regulatory protein VirG of the hairy-root-inducing plasmid pRiA4. *Gene* **78:**173-178.

8. **Axelsson, L.** 1998. Lactic acid bacteria: Classification and physiology. *in* S. Salminen and A. von Wright, Lactic Acid Bacteria, Microbiology and Functional Aspects. Marcel Dekker, New York.PP:1-73.

9. **Bacha, K., Machari, K. and Ashenafi, M.** 2010. Antimicrobial susceptibility patterns of LAB isolated from Walalim, A traditional Ethiopian fermented sausage. *J. Food Suppl.* **30:** 213-223.

10. **Baneyx, F.** 1999. Recombinant protein expression in *Escherichia coli. Curr. Opin. Biotechnol.* **10:**411-421.

11. **Barros, R. R., Carvalho, M. D. G. S., Peralta, J. M., Facklam, R. R. and Teixeira, L. M.** 2001. Phenotypic and Genotypic Characterization of *Pediococcus* Strains Isolated from Human Clinical Sources. *J. Clin. Microbiol.* **39:**1241-1246.

12. **Bjorkroth, J. and Holzapfel, W.** 2006. Genera *Leuconostoc, Oenococcus* and *Weissella. In* M. Dworkin (ed.), The prokaryotes: a handbook on the biology of bacteria: Firmicutes, Cyanobacteria. vol. 4, 3rd edition Springer-Verlag, New York, NY. PP:267-319.

13. **Bjorkroth, K. J., Schillinger, U., Geisen, R., Weiss, N., Hoste, B., Holzapfel, W. H., Korkeala, H. J. and Vandamme, P.** 2002. Taxonomic study of *Weissella confusa* and description of *Weissella cibaria* sp. nov.,detected in food and clinical samples. *Int. J. System. and Evol. Microbiol.* **52:**141–148.

14. **Boe, L., Gros, M.F., te Riele, H., Ehrlich, S.D. and Gruss, A.** 1989. Replication origins of single-stranded-DNA plasmid pUB110. *J. Bacteriol.* **171:**3366-3372.

15. **Braat, H., Rottiers, P., Hommes, D. W., Huyghebaert, N., Remaut, E., Remon, J. P., van Deventer, S. J., Neirynck, S., Peppelenbosch, M. P. and Steidler, L.** 2006. A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. *Clin. Gastroenterol. Hepatol.* 4:754–759.

16. **Byrd, D. R. and Matson, S. W.** 1997. Nicking by transesterification: the reaction catalyzed by a relaxase. *Mol. Microbiol.* **25:**1011-1022.

17. **Chen, Y. S., Yanagida, F. and Shinohara, T.** 2005. Isolation and identification of lactic acid bacteria from soil using an enrichment procedure. *Lett. Appl. Microbiol.* **40:**195-200.

18. **Choi, H. J., Cheigh, C. I., Kim, S. B., Lee, J. C., Lee, D. W., Choi, S. W., Park, J. M. and Pyun, Y. R.** 2002. *Weissella kimchii* sp. nov., a novel lactic acid bacterium from kimchi. *Int. J. Syst. Evol. Microbiol.***52:**507-511.

19. **Clark, A. J. and Warren, J.** 1979. Conjugal transmission of plasmids. *Annu. Rev. Genet.* **13:**99-125.

20. **Clewell, D. B.** 2007. Properties of Enterococcus faecalis plasmid pAD1, a member of a widely disseminated family of pheromone-responding, conjugative, virulence elements encoding cytolysin. *Plasmid* 58:205-227.

21. **Clewell, D. B. and Francia, M. V.** 2004. Conjugation in gram-positive bacteria. *In* B. E. Funnell and G. Phillips (eds), Plasmid biology. ASM Press, Washington, DC, PP:227-256.

22. **Cogan, T. M.** ***et al.,*** 1997. Characterization of the lactic acid bacteria in artisanal dairy products. *J. Dairy Res.* **64:**409-421.

23. **Collins, M. D., Samelis, J., Metaxopoulos, J. and Wallbanks, S. 1993.** Taxonomic studies on some *Leuconostoc*-like organisms from fermented sausages: description of a new genus *Weissella* for the *Leuconostoc paramesenteroides* group of species. *J. Appl. Bacteriol.* **75:**595-603.

24. **Datta, S., Larkin, C. and Schildbach, J. F.** 2003. Structural insights into single-stranded DNA binding and cleavage by F factor TraI. *Structure.* 11:1369–1379.

25. **del Solar, G., Giraldo, R., Ruiz-Echevarría, M. J., Espinosa, M. and Díaz-Orejas, R.** 1998. Replication and control of circular bacterial plasmids. *Microbiol. Mol. Biol. Rev.* **62:**434–464.

26. **del Solar, G., Moscoso, M. and Espinosa, M.** 1993. Rolling circle-replicating plasmids from gram-positive and -negative bacteria: a wall falls. *Mol. Microbiol.* **8:** 789-796.

27. **del Solar, G. H., Puyiet, A. and Espinosa, M.** 1987. Initiation signals for the conversion of single stranded to double stranded DNA forms in the streptococcal plasmid pLS1. *Nucl. Acids Res.* **15:**5561-5580.

28. **de Man, J. C., Rogosa, M. and Sharpe, M. E.** 1960. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* **23:**130–135.

29. **de Smit, M. H. and van Duin, J.** 1990. Secondary structure of the ribosome binding site determines translational efficiency: A quantitative analysis. *Proc. Natl. Acad. Sci.* USA. **87:** 7668-7672.

30. **Devriese, L. A., Van de Kerckhove, A., Kilpper-Balz, R. and Schleifer, K. H.** 1987. Characterization and Identification of Enterococcus Species Isolated from the Intestines of Animals. *Inter. J. Food Microbiol.* **37:**257-259.

31. **Dimov, S. G.** 2007. A Novel Bacteriocin-Like Substance Produced by *Enterococcus faecium* 3587. *Curr. Microbiol.* **55:**323-327.

32. **Ennahar, S., Cai, Y. and Fujita, Y.** 2003. Phylogenetic Diversity of Lactic Acid Bacteria Associated with Paddy Rice Silage as Determined by 16S Ribosomal DNA Analysis. *Appl. Environ. Microbiol.* **69:**444-451.

33. **Estrem, S. T., Gaal, T., Ross, W. and Gourse, R. L.** 1998. Identification of an UP element consensus sequence for bacterial promoters. *Proc. Natl. Acad. Sci.* USA. **95:** 9761-9766.

34. **EuropeanCommittee forAntimicrobial SusceptibilityTesting (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID)** 2000. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution Clinical Microbiology and Infection

35. **Facklam, R.** 2002. What happened to the streptococci: overview of taxonomic and nomenclature changes. *Clin. Microbiol. Rev.* **15:** 613–630.

36. **Fisher, K. and Phillips, C.** 2009. The ecology, epidemiology and virulence of Enterococcus". *Microbiol.* **155:**1749–1757.

37. **Freier, S.M., Kierzek, R., Jaeger, J. A., Sugimoto, N., Caruthers, M.H., Neilson, T. and Turner, D.H.** 1986. Improved free-energy parameters for predictions of RNA duplex stability. Proc. Natl. Acad. Sci. USA **83**:9373-937.

38. **Francia, M. V. and Clewell, D. B.** 2002. Amplification of the Tetracycline Resistance Determinant of pAMα1 in *Enterococcus faecalis* Requires a Site-Specific Recombination Event Involving Relaxase. *J. Bacteriol.* **184:**5187-5193.

39. **Francia, M. V., Varsaki, A., Garcillan-Barcia, M. P., Latorre, A., Drainas, C. and de la Cruz, F.** 2004. A classification schemes for mobilization regions of bacterial plasmids. *FEMS Microbiol. Rev.* **28:** 79-100.

40. **Fujimoto, S. and Ike, Y. 2001.** pAM401-based shuttle vectors that enable overexpression of promoterless genes and one-step purification of tag fusion proteins directly from *Enterococcus faecalis. Appl. Environ. Microbiol.* **67:**1262-1267.

41. **Garcia-Migura, L., Hasman, H. and Jensen, L. B.** 2009.Presence of pRI1: a small cryptic mobilizable plasmid isolated from Enterococcus faecium of human and animal origin. *Curr. Microbiol.* **58:**95-100.

42. **Garcillan-Barcia, M. P., Francia, M. V. and de la Cruz, F.** 2009. The diversity of conjugative relaxases and its application in plasmid classification. *FEMS Microbiol. Rev.* **33:** 657-687.

43. **Geary, C., Stevens, M., Sneath, P. H. A. and Mitchell, C. J.** 1989. Construction of a database to identify Staphylococcus species. *J. Clin. Pathol.* **42:**289-294.

44. **Geis, A.** 2003. Perspectives of genetic engineering of bacteria used in food fermentations. *in* Heller, K. J. (ed.), Genetically engineered food and methods of detection. Wiley-Vch, Weinheim PP:100-118.

45. **Gold, L.** 1988. Posttranscriptional regulatory mechanisms in *Escherichia coli*. *Annu. Rev. Biochem.* **57**:199-233.

46. **Gotz, F., Bannerrman, T. and Schliefer, K. H.** 2006. The genera *Staphylococcus* and *Macrococcus*. Prokaryotes **4:** 5-75

47. **Grohmann, E., Muth, G. and Espinosa, M.** 2003. Conjugative plasmid transfer in Gram-positive bacteria. *Microbiol. Mol. Biol. Rev.* **67**:277–301.

48. **Gros, M.-F., te Riele, H. and Ehrlich, S. D.** 1989. Replication origin of a single-stranded DNA plasmid pC194. *EMBO J.* **8**:2711-2716.

49. **Gruss, A. and Ehriich, S. D.** 1989. The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. *Microbiol. Rev.* **53**:231-241.

50. **Guasch, A., Lucas, M., Moncalián, G., Cabezas , M., Pérez-Luque, R., Gomis-Rüth, F. X., de la Cruz, F. and Coll, M.** 2003. Recognition and processing of the origin of transfer DNA by conjugative relaxase TrwC. *Nat. Struct. Biol.* **10**:1002–1010.

51. **Guzman, L. M. and Espinosa, M.** 1997. The mobilization protein, MobM, of the streptococcal plasmid pMV158 specifically cleaves supercoiled DNA at the plasmid oriT. *J. Mol. Biol.* **266**:688-702.

52. **Hang, M. J. and Lee, S. Y.** 2006. The *Escherichia coli* Proteome: Past, Present, and Future Prospects. *Microbiol. Mol. Biol. Rev.* **70**:362-439.

53. **Herranz, C., Casaus, P. and Mukhopadhyay, S. et al.** 2001. *Enterococcus faecium* P21: a strain occurring naturally in dry-fermented sausages producing the class II bacteriocins enterocin A and enterocin B. *Food Microbiol.* **18**:115-131.

54. **Ilyina, T. V. and Koonin, E. V.** 1992. Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria. *Nucl. Acids Res.* **20**:3279-3285.

55. **Jeong, D. W., Choi, Y. C., Lee, J. M., Kim, J. H., Lee, J. H., Kim, K. H. and Lee, H. J.** 2006. Isolation and characterization ofpromoters from Lactococcus lactis ssp. cremoris LM0230. Food Microbiol. **23**:82–89.

56. **Jeong, S. J., Park, J. Y., Lee, H. J. and Kim, J. H.** 2007. Characterization of pFMBL1, a small cryptic plasmid isolated from *Leuconostoc mesenteroides* SY2. *Plasmid* **57**:314-323.

57. **John, D. T. and James, H. J.** 1999. Antimicrobial Susceptibility testing: General Considerations. *in* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. Yolken (eds.), Manual of Clinical Microbiology. ASM, Washington DC PP:1469-1473.

58. **Johnston, L. M. and Jaykush, L. A.** 2004. Antimicrobial Resistance of *Enterococcus* Species Isolated from Produce. *Appl. Environ. Microbiol.* **70**:3133-3137.

59. **Kandler, O. and Weiss, N.** 1986. Regular, Nonsporing Gram Positive Rods. Genus Lactobacillus. *in* P. H. A. Sneath, N. S. Mair, M. E. Sharp, and J. G. Holt (eds.), Bergey's Manual of Systematic Bacteriology. The Williams & Wilkins Company, Baltimore MD PP: 1208-1234.

60. **Khan, S. A.** 1997. A rolling-circle replication of bacterial plasmids. *Microbiol. Mol. Biol. Rev.* **61**:442-455.

61. **Khan, S. A.** 2005. Plasmid rolling-circle replication: highlights of two decades of research. *Plasmid* **53**:126-136.

62. **Kilpper-Balz, R., Fiscer, G. and Schleifer, K. H.** 1982. Nucleic acid hybridization of group N and group D streptococci. *Curr. Microbiol.* 7:245-250.

63. **Kim, J. H. and Mills, D. A.** 2007. Improvement of a nisin-inducible expression vector for use in lactic acid bacteria. *Plasmid.* 58:275-283.

64. **Klein, G., Pack, A., Bonaparte, C. and Reuter, G.** 1998. Taxonomy and physiology of probiotic lactic acid bacteria. *Int. J. Food Microbiol.* **41**:103-125.

65. **Koort, J., Coenye, T., Vandamme, P., Sukura, A. and Bjorkroth, J.** 2004. *Enterococcus hermanniensis* sp. nov., from modified-atmosphere-packaged broiler meat and canine tonsils. *Int. J. Syst. Evol. Microbiol.* **54**:1823-1827.

66. **Kosinski, M. J., Rinas, U. and Bailey, J. E.** 1992. Isopropyl-beta-d-thiogalactopyranoside influences the metabolism of *E. coli*. *Appl. Microbiol. Biotechnol.* **36**:782-784.

67. **Kramer, M. G., del Solar, G. and Espinosa, M.** 1995. Lagging-strand origins of the promiscuous plasmid pMV158:physical and functional characterization. *Microbiol.* **141**:655-662.

68. **Kramer, M. G., Khan, S. A. and Espinosa, M.** 1997. Plasmid rolling circle replication: Identification of the RNA polymerase-directed primer RNA and requirement of DNA polymerase I for lagging strand synthesis. *EMBO J.* **16**:5784-5795.

69. **Kramer, M.G., Khan, S.A. and Espinosa, M.** 1998. Lagging strand replication from the ssoA origin of plasmid pMV158 in Streptococcus pneumoniae: in vivo and in vitro influences of mutations in two conserved ssoA regions. *J. Bacteriol.* **180**:83-89.

70. **Kramer, M. G., Espinosa, M., Mishra, T. K. and Khan, S. A.** 1999. Characterization of a single-strand origin, ssoU, required for broad host range replication of rolling-circle plasmids. *Mol. Microbiol.* **33**:466-475.

71. **Kurenbach, B., Bohn, C., Prabhu, J., Abudukerim, M., Szewzyk, U. and Grohmann, E.** 2003. Intergeneric transfer of the *Enterococcus faecalis* plasmid pIP501 to *Escherichia coli* and Streptomyces lividans and sequence analysis of its tra region. *Plasmid* **50:**86-93.

72. **Levkut M, Pistl J, Laukova A, *et al.*** 2009 Antimicrobial activity of *Enterococcus faecium* ef 55 against *Salmonella enteritidis* in chicks. *Acta Veterin. Hung.* **57:**13-24.

73. **Li, W., Li, H. X., Ji, S. Y., Li, S., Gong, Y. S., Yang, M. M. and Chen, Y. L.** 2007. Characterization of two temperature-inducible promoters newly isolated from *B. subtilis*. *Biochem. Biophysic. Res. Comm.* **358:**1148-1153.

74. **Lokman, B. C., Leer, R. J., Van Sorge, R. and Pouwels, P. H.** 1994. Promoter analysis and transcription regulation of Lactobacillus pentosus genes involved in xylose catabolism. *Mol. Gen. Genet.* **245:**117-125.

75. **Lorenzo-Diaz, F. and Espinosa, M.** 2009. Lagging-strand DNA replication origins are required for conjugal transfer of the promiscuous plasmid pMV158. *J. Bacteriol.* **191:** 720-727.

76. **Lorenzo-Diaz, F., Dostal, L., Coll, M., Schidbach, J. F., Menendez, M. and Espinosa, M.** 2011. The MobM relaxase domain of plasmid pMV158: thermal stability and activity upon Mn^{2+} and specific DNA binding. *Nucl. Asci. Res.* **39:** 4315-4329.

77. **Lourdes G. M., Henrik, H. and Lars, B. J.** 2009. Presence of pRI1: A small cryptic mobilizable plasmid isolated from Enterococcus faecium of human and animal origin. *Curr. Microbiol.* **58:**95-100.

78. **Madsen, S. M., Hindre, T., LePennec, J. P., Israelsen, H. and Dufour, A.** 2005. Two acid-inducible promoters from *Lactococcus lactis* require the *cis*-acting ACiD-box and the transcription regulator RcfB. *Mol. Microbiol.* **56:**735-746.

79. **Marelli, B. and Magni, C. H.** 2010. A simple expression system for *Lactococcus lactis* and *Enterococcus faecalis*. *World J. Microbiol. Biotechnol.* **26**:999-1007.

80. **Mills, S., McAuliffe, O. E., Coffey, A., Fitzgerald, G. F. and Ross, R. P.** 2006. Plasmids of lactococci-genetic accessories or genetic necessities? *FEMS Microbiol. Rev.* **30**:243-273.

81. **Miyoshi, A., Bermudez-Humaran, L. G., de Azevadto, M. S. P., Langella, P. and Azevedo, V.** 2010. Lactic Acid Bacteria as Live Vectors: Heterologous Protein Production and Delivery Systems. *In* F. Mozzi, R. Raya and G. M. Vignola. Biotechnology of lactic acid bacteria: novel applications. Wiley-Blackwell, Oxford, UK. **PP:**161-176.

82. **Monzingo, A. F., Ozburn, A., Xia, S., Meyer, R. J. and Robertus, J. D.** 2007. The structure of the minimal relaxase domain of MobA at 2.1 A resolution. *J. Mol. Biol.* **366:** 165-178.

83. **Morelli, L., Vogensen, F. K. and von Wright, A.** 2004. Genetics of lactic acid bacteria. *in* S. Salminen and A. von Wright (eds.), Lactic Acid Bacteria, Microbiology and Functional Aspects. Marcel Dekker, New York **PP:**249-293.

84. **Mundt, J. O.** 1986. Lactic Acid Streptococci. *in* P. H. A. Sneath, N. S. Mair, M. E. Sharp, and J. G. Holt (eds.), Bergey's Manual of Systematic Bacteriology. The Williams & Wilkins Company, Baltimore MD PP:1251-1264.

85. **Musikasang,H., Tani, A., H-kittikun, A. and Maneerat, S.** 2009. Probiotic potential of lactic acid bacteria isolated from chicken gastrointestinal digestive tract. *World J.Microbiol. Biotechnol.* **25:**1337-1345.

86. **Nash, R. P, Habibi, S., Cheng, Y., Lujan, S. A. and Redinbo, M. R.** 2010. The mechanism and control of DNA transfer by the conjugative relaxase of resistance plasmid pCU1. *Nucleic* **38:**5929–5943.

87. **Nauta, A., van Sinderen, D., Karsens, H., Smit, E., Venema, G. and Kok, J.** 1996. Inducible gene expression mediated by a repressor-operator system isolated from Lactococcus lactis bacteriophage r1t. *Mol. Microbiol.* **19**:1331-1341.

88. **Ndagano, D., Lamoureux, T., Dortu, C., Vandermoten, S. and Thonart, P.** 2011. Antifungal activity of 2 lactic acid bacteria of the Weissella genus isolated from food. *J. Food Sci.* **76**:305-311.

89. **Nes, I. F. and Johnsborg, O.** 2004. Exploration of antimicrobial potential in LAB by genomics. *Curr. Opin. Biotechnol.* **15**:100-104.

90. **Novick, R.P.** 1989. Staphylococcal plasmids and their replication. *Annu. Rev. Microbiol.* **43:** 537-565.

91. **Olano, A., Chua, J., Schroeder, S., Minari, A., Salvia, M. L. and Hall, G.** 2001. *Weissella confusa* (Basonym: *Lactobacillus confusus*) Bacteremia: a Case Report. *J. Clin. Microbiol.* **39**:1604-1607.

92. **Papagianni, M., Avramidis, N. and Filioussis, G.** 2007. High efficiency electrotransformation of *Lactococcus lactis* spp. *Lactis* cells pretreated with lithium acetate and dithiothreitol. *BMC Biotechnol.* **7**:15

93. **Parija, S. C.** 2009. Textbook of microbiology and immunology. Elsevier **PP.** 191-200.

94. **Platteeuw, C., Simons, G. and de Vos, W. M.** 1994. Use of the Escherichia coli beta-glucuronidase (gusA) gene as a reporter gene for analyzing promoters in lactic acid bacteria. *Appl. Environ. Microbiol.* **60**:587-593.

95. **Poyart, C. and Trieu-Cuot, P.** 1997. A broad host range mobilizable shuttle vector for the construction of transcriptional fusions to β-galactosidase in Gram-positive bacteria. *FEMS Microbiol. Lett.* **156**:193-198.

96. **Roberfroid, Marcel B.** 2000. Prebiotics and probiotics: are they functional foods? *Am. J. Clin. Nutr.* **71**:1682-1687.

97. **Rozen, S. and Skaletsky, H. J.** 2000. Primer3 on the WWW for general users and for biologist programmers. *In:* Krawetz, S. and Misener, S. (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, **PP:** 365-386.

98. **Rozkov, A. and Enfors, S. O.** 2004. Analysis and control of proteolysis of recombinant proteins in *Escherichia coli. Adv. Biochem. Engg. Biotechnol.* **89:**163-195.

99. **Saitou, H. and Miura, K.** 1963. Preparation of transforming deoxyribonucleicacid by phenol treatment. *Biochim. Biophys. Acta* **72:**619-629.

100. **Sakamoto, M., Uchimura, T. and Komagata, K.** 1996. Comparision og H2O forming NADH oxidase from *Leuconostoc mesenteroides* sub sp. *mesenteroides* NRIC 1541 and H2O2 forming NADH oxidase from *Sporolactobacillus inullinus* NRIC 1133. *J. Ferment. Bioengg.* **82:** 531-537.

101. **Sambrook, J., Fritsch, E. F. and Maniatis, T.** 1989. Molecular cloning: a Laboratory Manual, Cold Spring Harbor Laboratory press, Cold Spring Harbor, N.Y.

102. **Sanchez, C. and Mayo, B.** 2003. Sequence and analysis of pBM02, a novel RCR cryptic plasmid from Lactococcus lactis subsp cremoris P8-2-47. *Plasmid* **49:**118-129.

103. **Schleifer, K. H. and Kilpper-Balz, R.** 1987. Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci, and lactococci: a review. *Syst. Appl. Microbiol.***10:**1 - 9.

104. **Shareck, J., Choi, Y., Lee, B. and Miguez, C. B.** 2004. Cloning vectors based on cryptic plasmids isolated from lactic acid bacteria: their characteristics and potential applications in biotechnology. *Crit. Rev. Biotechnol.* **24:**155-208.

105. **Solaiman, D. K. Y. and Somkuti, G. A.** 1998. Characterization of a novel Streptococcus thermophilus rolling-circle plasmid used for vactor construction. *Appl. Microbiol. Biotechnol.* **50:**174-180.

106. **Stiles, M. E. and Holzapfel, W. H.** 1997. Lactic acid bacteria of foods and their current taxonomy. *Int. J. Food Microbiol.* **36:**1-29.

107. **Talwalkar, A. and Kailasapathy, K.** 2004. The role of oxygen in the viability of probiotic bacteria with reference to *Lactobacillus acidophilus* and Bifidobacterium spp. *Curr. Issues Intest. Microbiol.* **5:**1-8.

108. **Tarakanov, B. V., Iakovleva, A. A., Nikolicheva, T. A., Komkova, N. M., Manukhina, A. I. and Aleshin, V. V.** 2004. Expression vector pLF22 for the lactic acid bacteria. *Mikrobiolog.* **73:**211-217.

109. **te Riele, H., Michel, B. and Ehrlich, S. D.** 1986. Single stranded plasmid DNA in Bacillus subtilis and Staphylococcus aureus, *Proc. Natl. Acad. Sci.* **83:**2541-2545.

110. **Terzaghi, B. E. and Sandine, W. E.** 1975. Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol.* **29:**807-813.

111. **Teuber, M. and Geis, A.** 2006. The genus *lactococcus. in* M. Dworkin, S. Falkow, E. Rosenberg, *et al.* (eds), Springer science + Business media LLC, New York, PP:205-228.

112. **Todar, K.** 2009. Lactic Acid Bacteria. *in* K. Todar, Online Textbook of Bacteriology Wisconsin, university of Wisconsin Department of Biotechnology Madison **PP:**1-5.

113. **Toomey, N., Bolton, D. and Fanning, S.** 2010. Characterisation and transferability of antibiotic resistance genes from lactic acid bacteria isolated from Irish pork and beef abattoirs. *Res. Microbiol.***161:**127-135.

114. **Valdez-Cruz, N. A., Capseta, L., Perez, N. O., Ramirez, O. T. and Trujillo-Roldan, M. A.** 2010. Production of recombinant proteins in *E. coli* by the heat inducible expression system based on the phage lambda pL and/or pR promoters.*Microb. Cell Fact.* **9**:18.

115. **Van Rooijen, R. J., Gasson, M. J. and De Vos, W. M.** 1992. Characterization of the *Lactococcus lactis* Lactose operon promoter: contribution of flanking sequences and LacR repressor to promoter activity. *J. Bacteriol.* **174**:2273-2280.

116. **Voskuil, M. I. and Chambliss, G. H.** 1998. The -16 region of *Bacillus subtilis* and other gram-positive bacterial promoters. *Nucl. Asci. Res.* **26:** 2584-2590.

117. **Wang, A.N., Yi, X. W., Yu, H. F., Dong, B. and Qiao, S. Y.** 2009. Free radical scavenging activity of Lactobacillus fermentum in vitro and its antioxidative effect on growing–finishing pigs. *J. Appl. Microbiol.* **107:**1140-1148.

118. **Wardal, E., Sadowy, E. and Hryniewicz, W.** 2010. Complex nature of enterococcal pheromone responsive plasmids. *Polish J. Microbiol.* 59:79-87.

119. **Weaver, K. E., Rice, L. R. and Churchward, G.** 2002. Plasmids and transposons. *In* S. Gilmore (ed.), The enterococci: pathogenesis, molecular biology, and antibiotic resistance. ASM Press, Washington, DC, **PP:**219-263.

120. **Wood, B. J. B. and Holzapfel, W. H.** 1998. The genera of lactic acid bacteria. 1st edn. Blackie Academic & Professional, London.

121. **Wyckoff, H. A., Barnes, M., Gillies, K. O. and Sandine, W. E.** 1996. Characterization and sequence analysis of a stable cryptic plasmid from Enterococcus faecium 226 and development of a stable cloning vector. *Appl. Environ. Microbiol.* **62:**1481-1486.

122. **Yang, X. and McFadden, B. A.** 1993. A small plasmid, pCA2.4, from the cyanobacterium synechocystis sp. Strain PCC6803 encodes a Rep protein and replicates by a rolling circle mechanism. *J. Bacteriol.* **175:**3981-3991.

123. **Yeng, H. W., Mariana, N. S. and Raha, A. R.** 2009. Construction of an expression vector for *Lactococcuslactis* based on an indigenous cryptic plasmid. *African J. Biotechnol.* **8:**5621-5626.

124. **Zhou, X. X., Li, W. F., Ma, G. X. and Pan, Y. J.** 2006. The nisin-controlled gene expression system: construction, application and improvements. *Biotechnol. Adv.* **24:**285–295.

125. **Zhu, W.** ***et al.*** 2010. Dissemination of an *Enterococcus* Inc18-Like *vanA* Plasmid Associated with Vancomycin-Resistant *Staphylococcus aureus. Antimirob. Ag. Chemo.* 54:4314-4320.

126. **Zhu, Y., Zhang, Y. and Li, Y.** 2009. Understanding the industrial application potential of lactic acid bacteria through genomics. *Appl. Microbiol. Biotechnol.* **83:**597-610.

Appendix

Table A.1.1: PRIMERS OR OLIGONUCLEOTIDES

Si. No.	Primers Name	Primers sequences (5′ ---------- 3′)	Amplification for
1	**F8 - 27**	AGAGTTTGATCCTGGCTGAG	16S rDNA
2	**R1541 – R1522**	AAGGAGGTGATCCAGCCGCA	16S rDNA
3	**342 R**	CTGCTGCCTCCCGTAG	16S rDNA
4	**NJAKDF1**	CAGCTGAAAGGGGTTAGAATATATC	AKD1 promoter
5	**NJAKDR1**	CCATGGCGCCGAAACAGCTT	
6	**NJAKDF2**	CAGCTGAAAGGGGTTAGAATAT	AKD2 Promoter
7	**NJAKDR2**	CCATGGTTGAAATAATGCTTAGGTA	

Table A.2.1: BACTERIAL STRAINS AND PLASMIDS

Strains or Plasmids	Genotype and/or relevant features	Source or reference
Strains		
E. coli DH5α	F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	MTCC 1652
E. coli JM101	F'[traD36,proAB[+],lacI[q],lacZ, M15] SupE,thi,(lac-proAB)	MTCC 1668
E. coli BL21 (DE3)	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	MTCC 1679
E. faecium DJ1	Natural isolates, isolated from cow milk	This study
Plasmids		
pBS SK+	Amp^r, Cloning vector	Invitrogen, USA.
pNJAKD	Cryptic plasmid from *E. faecium* DJ1	This study
pEGFP	Amp^r, plasmid containing *eGFP* gene	BD Biosciences Clontech
PEGFPD1	pEGFP derivative containing AKD1 promoter	This study
$pEGFPD_2$	pEGFP derivative containing AKD2 promoter	This study
$pEGFPD_3$	pEGFP derivative containing AKD3 promoter	This study
$pEGFPD_4$	pEGFP derivative containing AKD4 promoter	This study
$pEGFPD_5$	pEGFP derivative containing AKD5 promoter	This study
$pEGFPD_6$	pEGFP derivative containing AKD6 promoter	This study
$pEGFPD_7$	pEGFP derivative containing AKD7 promoter	This study
pEGFPL	pEGFP derivative deleting lacP promoter and lacZ regions	This study
$pEGFPL_1$	pEGFPL derivative containing AKD1 promoter	This study
$pEGFPL_2$	pEGFPL derivative containing AKD2 promoter	This study
$pEGFPL_3$	pEGFPL derivative containing AKD3 promoter	This study
$pEGFPL_4$	pEGFPL derivative containing AKD4 promoter	This study
$pEGFPL_5$	pEGFPL derivative containing AKD5 promoter	This study
$pEGFPL_6$	pEGFPL derivative containing AKD6 promoter	This study
$pEGFPL_7$	pEGFPL derivative containing AKD7 promoter	This study

Enterococcus faecium strain DJ1 16S ribosomal RNA gene, partial sequence

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  AUTHORS   Kumar,N., Shridevi,R., Putarajan,A., Mahalaxmi,R., Roy,U.
            and Das,A.
  TITLE     Using 16S rRNA gene sequence analysis to identify milk-
            Originated Enterococcus isolates
  JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 1391)
  AUTHORS   Kumar,N., Roy,U. and Das,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-DEC-2009) Biological Sciences Group, Birla
            Institute of Technology and Science Pilani, FD - III,
            Pilani, Rajasthan 333031, India
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Enterococcus faecium strain DJ2 16S ribosomal RNA gene, partial sequence

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  TITLE     Using 16S rRNA gene sequence analysis to identify milk-
            Originated Enterococcus isolates
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  TITLE     Direct Submission
  JOURNAL   Submitted (25-DEC-2009) Biological Sciences Group, Birla
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Weissella confusa strain NKD1 16S ribosomal RNA gene, partial sequence

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  JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 349)
  AUTHORS   Kumar,N., Jyotsana,N., Paul,S., Mukherji,D. and Das,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (25-DEC-2009) Biological Sciences Group, Birla
            Institute of Technology and Science Pilani, FD - III,
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  TITLE     Probiotic potential of lactic acid bacteria isolated from
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Enterococcus faecium strain DJ3 16S ribosomal RNA gene, partial sequence

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REFERENCE   1 (bases 1 to 450)
  AUTHORS   Kumar,N. and Das,A.
  TITLE     Species identification of lactic acid bacteria by PCR
            amplification
  JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 450)
  AUTHORS   Kumar,N., Paul,S., Jyotsana,N. and Das,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (25-MAR-2011) Department of Biological Sciences,
            Birla Institute of Technology and Science, Pilani,FD - III,
            Pilani, Rajasthan 333031, India
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Staphylococcus epidermidis strain NJ1 16S ribosomal RNA gene, partial sequence

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  AUTHORS   Kumar,N. and Das,A.
  TITLE     Species identification of lactic acid bacteria by PCR
            amplification
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 336)
  AUTHORS   Kumar,N., Paul,S., Jyotsana,N. and Das,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (25-MAR-2011) Department of Biological Sciences,
            Birla Institute of Technology and Science, Pilani,FD - III,
            Pilani, Rajasthan 333031, India
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BUFFER AND STOCKS SOLUTION

Chemical Stocks

1M Tris-Cl

Dissolve 12.11 g of Tris base in 80 ml of H_2O. Adjust the pH to the desired value by adding concentrated HCl

pH	HCl
7.4	7 ml
7.6	6 ml
8.0	4.2 ml

Allow the solution to cool to room temperature before making final adjustments to pH. Adjust the volume of the solution to 100 ml with H_2O. Sterilize by autoclaving.

0.5 M EDTA (pH 8.0)

Add 18.61 g of disodium EDTA.2 H_2O to to 80 ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~ 20 g of NaOH pellet). Sterilize by autoclaving. The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to 8.0 by addition of NaOH.

SDS (20% w/v)

Dissolve 20 g of SDS in 90 ml of H_2O; incubate it at 37 °C in water bath until it dissolves completely. Adjust volume to 100 ml. Sterilization is not necessary.

10 N NaOH

The preparation of NaOH involves a highly exothermic reaction, which can cause breakage of glass containers; prepare this solution in plastic tube. Dissolve 4 g of NaOH to 8 m autoclaved Milli-Ql H_2O. When the pellets have dissolved completely, adjust the volume to 10 ml with autoclaved Milli-Ql H_2O. Store at room temperature. Sterilization is not required.

Glycerol (60% v/v)

Dissolve 6 ml of Glycerol in 4 ml of autoclaved Milli-Q H_2O. Sterilize the solution by passing it through 0.22 μm filter, store at 4 °C.

Sodium acetate (3 M, pH 5.2 and pH 7.0)

Dissolve 40.83 g of sodium acetate.3 H_2O in 80 ml of H_2O. Adjust the pH to 5.2 with glacial acetic acid or adjust the pH to 7.0 with dilute acetic acid. Adjust the volume 100 ml with H_2O. Sterilize by autoclaving.

Enzyme Stocks

Lysozyme (10 mg/ml)

Dissolve solid lysozyme at a concentration of 10 mg/ml in 10 mM Tris-Cl (pH 8.0) immediately before use.

Proteinase K (20 mg/ml)

Dissolve solid proteinase K at a concentration of 20 mg/ml in 10 mM Tris-Cl (pH 8.0). Divide the stock solution into small aliquots and store at -20 °C.

RNase A (10 mg/ml)

Dissolve solid RNase A to 10 mg/ml in TE (pH 7.6). Heat to 100 °C for 15 min. Cool slowly at room temperature. Store in aliquots at -20 °C.

Extraction/ Lysis Buffer

STE

10 M Tris - Cl (pH 8.0)

0.1M NaCl

1 mM EDTA (pH 8.0)

Or

Dissolve 0.584 g NaCl, 0.121 g Tris base and 0.037 g $Na_2EDTA.2\ H_2O$ into 80 ml H_2O. Adjust the pH 8.0 with HCl. Adjust the volume 100 ml with H_2O. Sterilize by autoclaving.

Buffer P1 (QIAGEN)

Dissolve 0.606 g Tris base, 0.372 g $Na_2EDTA.2\ H_2O$ in 80 ml H_2O Adjust the pH 8.0 with HCl. Adjust the volume 100 ml with H_2O

Buffer P2 (QIAGEN) or Solution II

0.2 N NaOH (Freshly diluted from a 10 N stock)

1% (w/v) SDS

Prepare solution II fresh, and use at room temperature

Or

Dissolve 0.8 g NaOH pellet in 95 ml H_2O, add 5 ml 20% SDS (w/v) solution. The final volume should be 100 ml.

Buffer P3 (QIAGEN) or Solution III (pH 5.5)

5 M potassium acetate	60.0 ml
Glacial acetic acid	11.5 ml
H_2O	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

Or

Dissolve 29.45 g potassium acetate in 50 ml H_2O. Adjust the pH to 5.5 with glacial acetic acid (~ 11.5 ml). Adjust the volume 100 ml with autoclaved Milli-Q H2O.

QBT

Dissolve 4.383 g NaCl, 1.046 g MOPS in 80 ml H_2O. Adjust the pH to 7.0 with NaOH. Add 15 ml pure iso propanol and 1.5 ml 10% triton X-100 solution (v/v). Adjust the volume 100 ml with autoclaved Milli-Q H2O.

QC

Dissolve 5.844 g NaCl, 1.046 g MOPS in 80 ml H_2O. Adjust the pH to 7.0 with NaOH. Add 15 ml pure isopropanol. Adjust the volume 100 ml with autoclaved Milli-Q H2O.

QF

Dissolve 7.305 g NaCl, 0.606 g Tris base in 80 ml H_2O. Adjust the pH to 8.5 with HCl. Add 15 ml pure isopropanol. Adjust the volume 100 ml with autoclaved Milli-Q H2O

Sucrose Buffer (pH 8.0)

Sucrose 6.7 g
50 mM Tris (pH 8.0)
1 mM EDTA (pH 8.0)
Ingredients dissolved in 100 ml of autoclaved Milli-Q H2O

EDTA Tris Buffer (pH 8.0)

0.25 M EDTA (pH 8.0)
50 mM Tris-Cl (pH 8.0)
Ingredients dissolved in autoclaved Milli-Q H2O

SDS Buffer

20% SDS (w/v)
50 mM Tris-Cl (pH 8.0)
20 mM EDTA
Ingredients dissolved in 100 ml autoclaved Milli-Q H2O

TE Buffer (pH 8.0)

10 mM Tris-Cl (pH 8.0)

1 mM EDTA (pH 8.0)

Electrophoresis Buffer/ Gel loading buffer

50X TAE (pH ~ 8.3, for 1000 ml)

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

5X TBE (pH ~ 8.3, for 1000 ml)

Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml

6X Gel loading buffer (store at 4 °C)

0.25% (w/v) Bromophenol blue

0.25% (w/v) Xylene cyanol FF

40% (w/v) Sucrose in water

Hybridization Buffer

Alkaline Transfer Buffer

0.4 N NaOH
1 M NaCl

Denaturation Solution (for neutral transfer, ds-DNA targets only)

1.5 M NaCl
0.5 M NaOH

Neutralization Buffer

0.5 M Tris-Cl (pH 7.2)
1 M NaCl

Neutralizing solution

0.5 M Tris-Cl (pH 7.0)
1.5 M NaCl

20X SSC

Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of autoclaved Milli-Q H_2O. Adjust the pH 7.0 with few drops of a concentrated HCl. Adjust the volume to 1000 ml with autoclaved Milli-Q H_2O. Sterilize by autoclaving. The final concentrations of the ingredients are 3.0 M NaCl and 0.3 M sodium citrate.

DNA MODIFYING ENZYMES

T4 DNA Ligase (MBI Fermentas)

T4 DNA Ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA or RNA. The enzyme repairs single-strand nicks in duplex DNA, RNA or DNA/RNA hybrids, joins DNA fragments with either cohesive or blunt termini, but has no activity on single-stranded nucleic acids.The T4 DNA Ligase requires ATP as cofactor.

T4 Polynucleotide Kinase (T4 PNK, MBI Fermentas)

T4 Polynucleotide Kinase (T4 PNK) catalyzes the transfer of the gamma-phosphate from ATP to the 5'-OH group of single- and double-stranded DNAs and RNAs, oligonucleotides or nucleoside 3'-monophosphates (forward reaction). The reaction is reversible. In the presence of ADP T4 Polynucleotide Kinase exhibits 5'-phosphatase activity and catalyzes the exchange of phosphate group between 5'-P-oligo-polynucleotides and ATP (exchange reaction). The enzyme is also a 3'-phosphatase.

Taq DNA polymerase (Banglore Genei)

Taq DNA polymerase is a highly thermostable DNA polymerase of the thermophilic bacterium *Thermus aquaticus*. The enzyme catalyzes 5'=>3' synthesis of DNA, has no detectable 3'=>5' exonuclease (proofreading) activity and possesses low 5'=>3' exonuclease activity. In addition, *Taq* DNA polymerase exhibits deoxynucleotidyl transferase activity, which frequently results in the addition of extra adenines at the 3'-end of PCR products.

T4 DNA Polymerase (MBI Fermentas)

T4 DNA Polymerase, a template-dependent DNA polymerase, catalyzes 5'=>3' synthesis from primed single-stranded DNA. The enzyme has a 3'=>5' exonuclease activity, but lacks 5'=>3' exonuclease activity.

S1 Nuclease (MBI Fermentas)

S1 Nuclease degrades single-stranded nucleic acids, releasing 5'-phosphoryl mono- or oligonucleotides, S1 Nuclease also cleaves dsDNA at the single-stranded region caused by a nick, gap, mismatch or loop. S1 Nuclease exhibits 3'-phosphomonoesterase activity.

CULTURE MEDIA

LB Broth, Miller (Luria-Bertani Broth, HIMEDIA)

Ingredients	Gms/litre
Casein enzymic hydrolysate	10.00
Yeast extract	5.00
NaCl	10.00

Final pH 7.5 ± 0.2

Suspend 25.0 g in 1000 ml distilled water. Sterilize by autoclaving

Lactobacillus MRS Broth (MRS Broth, HIMEDIA)

Ingredients	Gms/litre
Protease peptone	10.00
Beef extract	10.00
Yeast extract	5.00
Dextrose	20.00
Tween – 80	1.00
Ammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulphate	0.10
Manganese sulphate	0.05
Dipotassium phosphate	2.00

Final pH 6.5 ± 0.2

Suspend 55.15 g in 1000 ml distilled water. Sterilize by autoclaving

M17 Medium

Ingredients	Gms/litre
Peptone	5.00
Tryptone	5.00
Beef extract	5.00
Yeast extract	2.50
Ascorbic acid	0.50
Sodium - β - glycerophosphate	1.00
$MgSO_4$	0.25
Glucose	5.55

Dissolve in 1000 ml distilled water. Adjust pH 7.0, Sterilize by autoclaving

Sodium Acetate Skim Milk Dextrose Medium (SSMD Medium)

Ingredients	Gms/litre
Yeast extract	5.00
Peptone	5.00
Sodium acetate	10.00
Dextrose	10.00
Skim Milk	10.00

Dissolve in 1000 ml distilled water. Adjust pH 6.8, Sterilize by autoclaving

List of Publications and Patent

Kumar, N., Jyotsana, N., Paul, S., and Das, A. 2011. Identification and screening of lactic acid bacteria for the presence of naturally occurring plamids. *International Journal of Integrative Biology* **11(2):** 85 - 89.

Kumar, N., Ponnaluri, C. V. K., Putarjunan, A., Ranganathan, S., Roy, U., and Das, A. 2011. Characterization of temperature inducible promoters from a novel rolling circle replicating plasmid of *Enterococcus faecium* DJ1. **(Plasmid; In Press)**

Mehrotra, R., Gupta, G., Sethi, R., Bhalothia, P., **Kumar, N.,** Mehrotra, S. 2011. Designer promoter: an artwork of cis engineering. *Plant Molecular Biology* 75(6): 527 - 536.

3004/DEL/2010, **Das, A. and Kumar, N.** 2010. Temperature inducible promoter derivable from *Enterococcus faecium* DJ1 plasmid, and its use in *E. coli* for production of desired protein

List of Paper and Posters presented in Conferences

Posters:

Kumar, N., Saxena, V., Garg, S., Ponnaluri, C.V.K., and Das, A. 2006. Characterization of plasmid from natural isolates of lactic acid bacteria. All India Seminar on Advances in Botanical, Biotechnological & Microbiological Researches (AISABBMR), University of Bikaner & M.N. Institute of Applied Sciences, Bikaner, 26^{th} – 28^{th} Dec. pg. 140.

Kumar, N., **Jyotasana, N.,** Roy, U., and Das, A., 2009. Characterization of naturally isolated latic acid bacteria strains. National Seminar on Frontiers in Biotechnolgy (NSFB), Department of Biotechnology, Bharathiar University, Coimbatore, 22^{nd} - 24^{th} July pg. 55

Kumar, N., Ponnaluri, C.V.K., Ramdoss, M., Putarajan, A., Kayalvizhi, M., Dutta, A., Mukherjee, D., Subramaniyam, N., and Das, A. 2007. Characterization of plasmid from natural isolates of lactic acid bacteria and analyses to identify and screen novel Promoters. International Symposium on Applied Phycology and Environmental Biotechnology, Biological Science Group and Centre for Desert Development Technologies, Birla Institute of Technology and Science, Pilani, 29^{th} – 31^{st} Oct. pg. 78.

Kumar, N., Ponnaluri, C.V.K., Ramdoss, M., Putarajan, Dutta, A., Roy, U., and Das, A. 2011. Characterization of temperature inducible promoters from plasmids of natural isolates of lactic acid bacteria. National Conferences on Contemporary Trends in Biological and Pharmaceutical Research (CTBPR), Department of Biological Sciences and Department of Pharmacy, Birla Institute of Technology and Science, Pilani, 12^{th} -13^{th} March, pg. 66.

Paper Presentation

Kumar, N., Roy, U., Jyotasana, N., and Das, A., 2009. Characterization of a novel rolling circle replication cryptic plasmid pNJAKD from *Enterococcus faecium* Strain DJ1. National Seminar on Frontiers in Biotechnolgy (NSFB), Department of Biotechnology, Bharathiar University, Coimbatore, 22^{nd} - 24^{th} July, pg. 83.

Biography of Prof. Ashis K. Das

Prof. Ashis K. Das is presently the Dean, R & C Division and Professor, Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, Rajasthan. He received his PhD. degree in 1993 from National Institute of Immunology (Jawaharlal Nehru University), New Delhi. He has been a Post Doctoral Fellow in the Department of Molecular Biology and Immunology, SHPH, John Hopkins University, Baltimore, USA and a WHO Fellow at the Malaria Branch, Centers for Disease Control & Prevention, Atlanta, Georgia USA. He has been involved in teaching and research for 26 years. His areas of interest include Molecular and Immuno-Parasitology, Molecular Diagnostics and Industry oriented projects dealing with different aspects of Vector design, Cloning, Expression and Bioinformatics etc. He has published various research articles in peer reviewed international journals. He has been PI for projects funded by CSIR, DBT, UGC and industry.

Biography of Mr. Narayan Kumar

Mr. Narayan Kumar completed his B. Sc. Chemistry degree from Banaras Hindu University, Varanasi in 2002 and M. Sc. Biotechnology from Indian Institute of Technology, Roorkee in 2004. He worked as a chemist in Techno concepts PVT. India Ltd. He received all over India 20th rank ((99.54 percentile) in Graduate aptitude test examination. He has been enrolled as Doctoral fellow at Birla Institute of Technology and Science, Pilani, since Jan, 2005. His areas of interest include Biochemistry and Molecular Biology aspects of Lactic acid bacteria, Plasmid manipulation and Prokaryotic vector designing. His research work was supported with institute research scholarship and UGC-BSR fellowship. He has published research articles in well renowned international journals. A patent on Temperature inducible Novel Promoter has been filed which includes his name as an inventor. He also received best poster award on Characterization of Naturally isolated Latic acid Bacteria Strains in National Seminar on Frontiers in Biotechnolgy (NSFB) 2009, organized by Department of Biotechnology, Bharathiar University, Coimbatore.