The Role of Transcription in Lactococcal Phage Replication

A Thesis Presented in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Microbiology

at Massey University, Palmerston North, New Zealand

Anja Heike Schiemann

February 2003

Massey University
COLLEGE OF SCIENCES

Institute of Molecular BioSciences
Private Bag 11 222,
Palmerston North,
New Zealand
Telephone: 64 6 356 9099
Facsimile: 64 6 350 5688
Email: IMBS@massey.ac.nz

CERTIFICATE OF REGULATORY COMPLIANCE

This is to certify that the research carried out in the Doctoral Thesis entitled "The role of transcription in lactococcal phage replication" in the Institute of Molecular BioSciences at Massey University, New Zealand:

(a) is the original work of the candidate, except as indicated by appropriate attribution in the text and/or in the acknowledgements;
(b) that the text, excluding appendices/annexes, does not exceed 100,000 words:
(c) all the ethical requirements applicable to this study have been complied with as required by Massey University, other organizations and/or committees which had a particular association with this study, and relevant legislation.

Please insert Ethical Authorisation code(s) here: (if applicable) _____
ERMA ISBC GM0 99 / MU14

Candidate's Name: Anja Heike Schiemann
Signature: Anja Schiemann
Date: 2/2/2003

Supervisor's Name: Paul W. O'Toole
Signature: Paul O'Toole
Date: 14/1/2003

Massey University
COLLEGE OF SCIENCES

Institute of Molecular
BioSciences
Private Bag 11 222,
Palmerston North,
New Zealand
Telephone: 64 6 356 9099
Facsimile: 64 6 350 5688
Email: IMBS@massey.ac.nz

Date: 14/1/2003

TO WHOM IT MAY CONCERN

This is to state that the research carried out for the Doctoral thesis entitled "The role of transcription in lactococcal phage replication" was done by Anja Heike Schiemann in the Institute of Molecular BioSciences at Massey University, Palmerston North, New Zealand. This thesis material has not been used in part or in whole for any other qualification, and I confirm that the candidate has pursued the course of study in accordance with the requirements of the Massey Univeristy regulations.

Supervisor's Name Paul W. O'Toole

Signature Paul W. O'Toole

Data 14/1/2003

Massey University
COLLEGE OF SCIENCES

Institute of Molecular BioSciences
Private Bag 11 222,
Palmerston North,
New Zealand
Telephone: 64 6 356 9099
Facsimile: 64 6 350 5688
Email: IMBS@massey.ac.nz

CANDIDATE'S DECLARATION

This is to certify that the research carried out for my Doctoral thesis entitled "The role of transcription in lactococcal phage replication" in the Institute of Molecular BioSciences, Massey University, Palmerston North, New Zealand is my own work and that the thesis material has not been used in part or in whole for any other qualification.

Candidate's Name Anja Heike Schiemann

Signature Anja Schiemann

Date 2/2/03

Abstract

The involvement of transcription in DNA replication has been demonstrated in mitochondria, bacteria, plasmids and phages. Previous studies indicated that transcription may also be required for the prolate-headed lactococcal phage c2 DNA replication in *L. lactis*.

In this study, the role of transcription in origin of DNA replication function was examined. A model system was used, in which the intergenic region of phage c2, presumably containing the origin of DNA replication, supported the replication of a plasmid in *Lactococcus lactis*. Within this region there is an active promoter that produces several non-coding transcripts. This allowed the importance of this early promoter 1 (P_E1) and the length and sequence (and presumably therefore secondary structure) of the P_E1 transcripts in replication to be investigated. It was demonstrated that a functional promoter (but not necessarily wildtype P_E1) and a specific length and sequence of the P_E1 transcripts are required for c2 origin function.

The transcription start site of the P_E1 transcripts in the plasmid system was determined by primer extension analysis and was identical to the transcription start site in the phage itself. The P_E1 transcripts made in the replicating plasmids were detected and quantified by Northern blots, and processing of the transcripts was shown by RNase protection analysis. However, no transcripts of the expected size were detected in the non-functional origins cloned in a plasmid able to replicate in *L. lactis*. Possible secondary structures of the wildtype and modified P_E1 transcripts were modelled using several different computer programs.

Lactococcal proteins were shown to bind to the P_E1 transcripts by RNA gel shifts and North-Western blots. Affinity purification and amino-terminal sequencing were used to identify one such protein with similarity to the ribosomal protein S1.

Growth curves of *L. lactis* containing the various replicating plasmids did not show any major differences in the ability of the *L. lactis* cells to grow. To characterize the plasmids further, the relative amount of plasmid DNA per lactococcal cell was determined. It was also demonstrated, by using a high copy number plasmid that harboured the c2 origin, that the c2 origin does not confer a Per phenotype.

Acknowledgments

I would like very much to thank my chief supervisor Dr Paul O'Toole who guided me through the whole project, advising and encouraging me during my studies and who even after shifting to the "other side" of the world always responded promptly to my e-mails.

I am also very grateful to my second supervisor, Dr Mark Lubbers (Fonterra Research Centre), for his constant support during this work and who found solutions to many problems due to his broad knowledge about lactic acid bacteria.

I also want to thank Dr Jasna Rakonjac who was always available to discuss experiments and results and most importantly, who always cheered me up if things did not go the way I wanted.

I want to thank all the members of the Helipad who made life and work much more enjoyable: Tash was a great lab organizer and taught me the "kiwi language", with Millis I could always talk about the strange kiwi habits and language, and with Mikara I had my morning chats about everything that was going on in the world. Also thanks to Jakki, Todd and Pania who made the lab a good place to work and Michael who held out with me when everybody else had already deserted the Helipad. I must also not forget Steffen, who introduced me to a lot of people who became my friends when I first came to Palmerston North and did not know anybody.

I want to thank all the people from the Institute of Molecular BioSciences who supported my work by allowing me to use their equipment, performing the DNA and amino acid sequencing and giving advice with experiments. Thanks especially to Paul Gardner, who helped me with the RNA secondary structure modelling and to Dr Rissa Ota, who was a big help with the statistical analysis.

I gratefully acknowledge the financial support from the award of a Massey University Doctoral Scholarship, the Marsden Fund of the Royal Society of New Zealand, and the New Zealand Foundation for Research, Science and Technology.

Travel funding was gratefully received from the Institute of Molecular BioSciences at Massey University.

Thanks to all my friends in Palmerston North who supported me in various ways during my studies, especially Delwyn who was not only my flatmate for quite some time and always a good friend, but also proofread my thesis. Also thanks to my friends in Germany who didn't forget me even though I have not seen many of them for years.

I also want to thank my partner Kim, who always supported me and had to put up with me during the last years, when I was grumpy and stressed because of experiments not working and the thesis writing progress being too slow.

Besonderen Dank an meine Familie, die mich immer unterstützt hat obwohl ich auf der anderen Seite der Erde war und ohne die diese Arbeit nicht möglich gewesen wäre.

Abbreviations

All abbreviations and units used in this thesis and not specified in this list are standard SI-units.

aa	amino acid
ABI	Applied Biosystems
amp	ampicillin resistance gene
APS	ammonium persulphate
AT	content of deoxyadenylate and deoxythymidylate in DNA
ATP	adenosine triphosphate
BHI-A	brain heart infusion agar
BHI-B	brain heart infusion broth
BLAST	basic local alignment search tool
BSA	bovine serum albumin
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
cDNA	complementary DNA
cfu	colony forming units
cm	chloramphenicol resistance gene
cos	cohesive
cpm	counts per minute
CTP	cytidine triphosphate
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
ds	double-stranded
DTT	DL-dithiothreitol
ECL	enhanced chemiluminescence (Amersham)
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic migration shift assay
ery	erythromycin resistance gene

FP	forward primer
g	acceleration equivalent to the earth's gravity (9.806 ms^{-2})
G+C	content of deoxyguanylate and deoxycytidylate in DNA
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
IPTG	isopropyl-β-D-thiogalactopyranoside
kan	kanamycin resistance gene
LB	Luria Bertani broth
LBA	Luria Bertani agar
MCS	multiple cloning site
MIC	minimum inhibitory concentration
MOPS	3-(N-morpholino) propanesulphonic acid
mRNA	messenger RNA
n/a	not applicable
OD	optical density at specified wavelength in nanometers
ON	overnight
ORF	open reading frame
ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Per	phage encoded resistance
pfu	plaque forming units
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
Pos.	position
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RNase	ribonuclease
RP	reverse primer
rpm	revolutions per minute
RT	room temperature
S.D.	standard deviation
SDS	sodium dodecyl sulphate
ss	single-stranded

SSC	saline-sodium citrate buffer
TBE	Tris borate EDTA buffer
TE	Tris EDTA buffer
TEMED	N,N,N',N' tetramethylethylenediamine
tet	tetracycline resistance gene
T_m	melting temperature
Tris	Tris(hydroxymethyl)methylamine
tRNA	transfer RNA
U	unit
UTP	uridine triphosphate
UV	ultraviolet (light)
vol	volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
ΔG	amount of energy required for or released by a reaction [Kcal/mol]
Ω	transcriptional and translational terminator derived from bacteriophage T4 gene

In addition, the conventional one-letter codes for amino acids, deoxyribonucleosides and ribonucleosides were applied:

amino acids: A, R, N, D, C, E, Q, G, H, I, L, K, M, F, P, S, T, W, Y, V for alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine respectively.

deoxyribonucleosides: A, C, G, T for deoxyadenylate, deoxycytidylate, deoxyguanylate and deoxythymidylate respectively.

ribonucleosides: A, C, G, U for adenylate, cytidylate, guanylate and uridylate respectively.

Table of Contents

List of Figures

List of Tables

1. INTRODUCTION

Bacterial strains of the genus *Lactococcus*, are commonly used in the manufacture of cultured milk products. Bacteriophage attack of lactococcal starter cultures very often results in lysis of susceptible strains causing fermentation failure and substantial economic loss for the dairy industry.

1.1 Bacteriophages

Bacteriophages are viruses that infect bacteria, and they have colonized every natural habitat tested to date. Rather than occurring free in the environment, many bacteriophages are found as prophages in lysogenic hosts. There are approximately 4-6 x 10^{30} prokaryotic cells in the biosphere (Whitman *et al.*, 1998) and it has been shown by direct counts on environmental samples that there are ~ 10 fold more tailed bacteriophage particles than cells (up to 2.5 x 10^{8} virus particles per ml natural water, Bergh *et al.*, 1989). Thus the total number of phages is enormous. Since 1959 over 5100 different phage types have been examined by electron microscopy (reviewed in Ackermann, 2001) and every year there are around 150 new phage discoveries. About 96% of all examined phages (4950 phages) are tailed, the remaining ~ 4% (186 phages) are cubic, filamentous, or pleomorphic.

1.1.1 A brief history

Bacteriophages were discovered independently by Frederick Twort (1915) and by Félix D'Herelle (1917), and were subsequently named "bacteriophages" (bacteria eaters). In 1934 D'Herelle and George Eliava co-founded an institute for phage research in the Soviet Republic of Georgia, where they worked on the therapeutic use of bacteriophages. However, with the discovery of penicillin and other chemical antibiotics in the 1940's, the Western world was no longer interested in phage therapy.

The modern era of bacteriophage research is usually dated from 1938 when Max Delbrück began his work on phages at the California Institute of Technology. In 1945 he started a training course at Cold Spring Harbor for phage biology. Salvadore Luria and Al Hershey soon joined Delbrück at Cold Spring Harbor thus continuing bacteriophage research as a route to understand basic features of biological life. During the 1950's and 1960's phage research had a dominant role in answering some of the most fundamental questions about what genes are and how their information determines the characteristics of an organism.

Dogmas central to biology that have originated from phage research are many. A few examples include the following: the discovery that DNA is the genetic material (Hershey and Chase, 1952), that mRNA is the intermediate for translating genetic information from DNA into protein (Volkin and Astrachan, 1956), that the genetic code is determined by three, nonoverlapping nucleotides (Crick *et al.*, 1961), that DNA replication is initiated from a complex of proteins and a nucleotide primer (Goulian and Kornberg, 1967), and that protein chaperonins facilitate protein folding (Georgopoulos *et al.*, 1973). Other findings include the basis of transcriptional regulation (Ptashne, 1967; Botstein *et al.*, 1975), regulation of gene expression (Roberts, 1969; Guarneros and Galindo, 1979), and restriction and modification systems (Luria and Human, 1952). Many methods which are commonly used in laboratories today originate from phage research, including gene cloning (Lobban and Kaiser, 1973) and protein separation by SDS-PAGE (SDS-polyacrylamide gel electrophoresis; Laemmli, 1970; Studier, 1973).

Phages and phage biology remains interesting in modern times for a number of reasons. Some bacteria are only pathogenic due to the temperate phages that they harbour (for example *V. cholerae;* Waldor and Mekalanos, 1996; for review see Miao and Miller, 1999). In the laboratory phages can be used for the display of antibody fragments and functional proteins such as protein and peptide hormones, and enzymes (phage display; Smith, 1985). With the rise of multidrug-resistant bacterial pathogens (including vancomycin resistant strains) there is new interest in phage therapy. Examples of some modern phage therapies that are currently being trialed include using

phages to kill harmful bacteria like enterococci (Biswas *et al.*, 2002) and using bacteriophage cell wall hydrolase to kill streptococci (Loeffler *et al.*, 2001).

1.2 Lactococcal bacteriophages

1.2.1 Bacteriophage attack of lactococci

Lactococcus lactis was the first bacterium described from a pure culture (Lister, 1873). Lactococci are AT-rich, coccoid, Gram-positive, anaerobic bacteria (formerly known as mesophilic lactic streptococci; Schleifer, 1987; for review see Konings *et al.*, 2000). Lactococci do not have flagella, nor do they form endospores. Recently, the genome of *L. lactis* IL1403 has been completely sequenced (Bolotin *et al.*, 1999; 2001). Lactococci are widely used in the manufacture of fermented dairy products (namely soft and hard cheeses, quark, cultured butter, sour milk, buttermilk, sour cream, and kefir) where their main function is to ferment lactose to lactic acid. The metabolic conversions involved in this process have been established and include the lactose phosphotransferase system (PTS), the tagatose-6-phosphate pathway and the glycolytic pathway (Gasson and de Vos, 1994).

Bacteriophages infect over 140 bacterial genera. So far, 814 phages have been examined in enterobacteria, 652 in lactococci and 412 in *Bacillus* (reviewed in Ackermann, 2001). The high number of lactococcal phages discovered is probably due to the extensive usage of lactococci in the dairy industry and the attention the phages have received due to their impact on industrial processes. Lysis of susceptible strains after phage exposure during the manufacturing process is a common cause of fermentation failure. Fermentation failure due to phage attack during the production of dairy products has been known for over 60 years (Whitehead and Cox, 1935) and is a source of economic loss for the industry. Improvement of hygiene in dairy plants, and starter strain selection and management, has led to a decrease of phage infection during the fermentation process in New Zealand. However, fermentation failure due to phage attack is still a major concern in many dairy plants worldwide.

1.2.2 Classification of lactococcal bacteriophages

Bacteriophages belong to 13 virus families, and are classified according to their morphology (cubic, helical or pleomorphic), tails and nucleic acid (Figure 1.1 and Table 1.1). Initially, lactococcal phages were differentiated by host range and resistance groups (Chopin *et al.*, 1976), serology (Jarvis, 1977) and morphology.

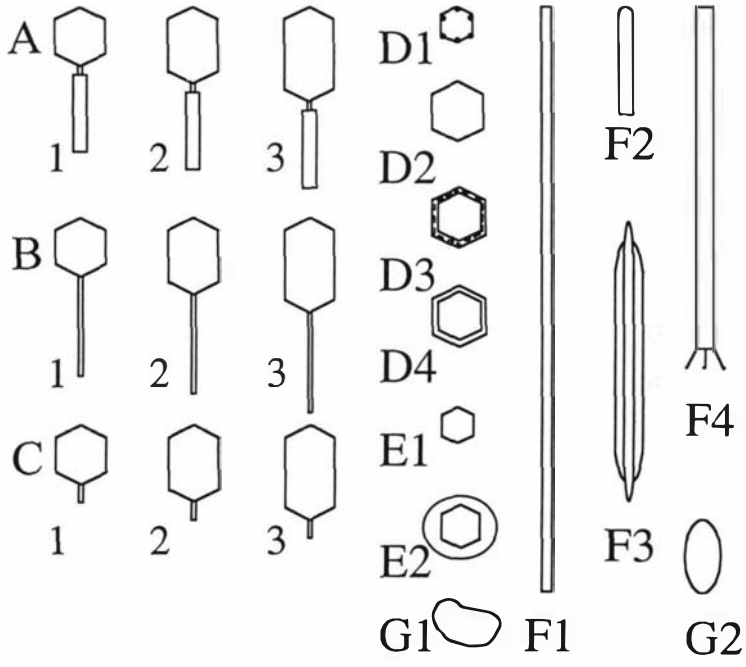


Figure 1.1. Morphotypes of bacteriophages.

Phages A1-C3 are tailed, D1-D4, and E1 and E2 are polyhedral, phages F1-F4 are filamentous, and G1 and G2 are pleomorphic (see Table 1.1 for details). Figure reproduced and modified from Ackermann (2001).

During recent years the molecular biology of lactococcal phages has been studied using a range of techniques, including DNA hybridization by Southern blot and heteroduplex analysis (Jarvis, 1984), genome sequencing, restriction endonuclease cleavage, and analysis of proteins by SDS-PAGE (reviewed in Jarvis *et al.*, 1991). Most

recently, multiplex PCR has been adapted for the detection and the identification of lactococcal phages (Labrie and Moineau, 2000).

Table 1.1. Morphotypes and basic properties of phage families.

Morphotype	Shape	Nucleic acid	Family	Particulars	Examples
A1 to A3	tailed	double-stranded, linear DNA	*Myoviridae*	contractile tail	T4, P1, P2, Mu
B1 to B3	tailed	double-stranded, linear DNA	*Siphoviridae*	long, noncontractile tail	λ, T1, T5, lactococcal phage c2
C1 to C3	tailed	double-stranded, linear DNA	*Podoviridae*	short tail	T7, ϕ29
D1	polyhedral	single-stranded, circular DNA	*Microviridae*	conspicuous capsomers	ϕX174
D3	polyhedral	double-stranded, circular, superhelical DNA	*Corticoviridae*	complex capsid, lipids	
D4	polyhedral	double-stranded, linear DNA	*Tectiviridae*	lipid vesicle, pseudotail	
E1	polyhedral	single-stranded, linear RNA	*Leviviridae*		MS2, Qβ
E2	polyhedral	double-stranded, linear, segmented RNA	*Cystoviridae*		
F1	filamentous	single-stranded, circular DNA	*Inoviridae*	long filaments	M13, f1, fd
F2	filamentous	single-stranded, circular DNA	*Inoviridae*	short rods	
F3	filamentous	double-stranded, linear DNA	*Lipothrixviridae*	envelope, lipids	
F4	filamentous	double-stranded, linear DNA	*Rudiviridae*	TMV-like	
G1	pleomorphic	double-stranded, circular, segmented DNA	*Plasmaviridae*	envelopes, lipids, no capsid	
G2	pleomorphic	double-stranded, circular, segmented DNA	*Fuselloviriade*	envelopes, lipids, no capsid, lemon-shaped	

Table modified from Ackermann (2001) and Maniloff and Ackermann (1998).

Morphotype D2 is for poorly known polyhedral phages awaiting classification.

Lactococcal phages are all tailed phages with linear double-stranded DNA, and have been classified into 12 species (Jarvis *et al.*, 1991). Electron microscopy of phages isolated from dairy fermentations has shown that nearly all lactococcal phages have an isometric or prolate polyhedral head and a noncontractile tail with or without a collar (Heap and Jarvis, 1980; Saxelin *et al.*, 1986; Teuber and Lembke, 1983). The lactococcal phages belong to the *Siphoviridae*, *Podoviridae,* and *Myoviridae* families (for more detail see Table 1.2) of the *Caudovirales* order (tailed phages, linear dsDNA genome; Maniloff and Ackermann, 1998; Ackermann, 1999). Most fermentation failures are due to the action of three genetically unrelated groups of lytic lactococcal phages of the *Siphoviridae* family (936, P335 and c2; Jarvis *et al.*, 1991; Moineau *et al.*, 1996).

Table 1.2. Species and type phages of lactococcal bacteriophages.

Family	Morphotype	Phage species	Type phage	Members
Siphoviridae	B1	936	P008	sk1, bIL66, biL41, p2, Q7, Q11, F4-1, US3
	B1	P335	P335	ul36, r1t, ϕLC3, P002, Tuc2009, TP901-1, ϕ31
	B1	P107	P107	
	B1	1483	1483	
	B1	P087	P087	
	B1	1358	1358	1404
	B1	BK5-T	BK5-T	
	B1	949	949	877, 1283, 1299
	B2	c2	c6A	c2, bIL67, P001, P6, Q38, c21, eb1, ml3, Q44, ϕvML3, 923
Podoviridae	C1	P034	P034	P369
	C3	KSY1	KSY1	
Myoviridae	A1			Rzh

Table modified from Jarvis *et al.* (1991).

These phages conform to one of two morphotypes: phages of the B1 morphotype which are characterized by small isometric heads and long noncontractile tails (936- and P335-like phages), and phages of the B2 morphotype which are characterized by prolate heads and long noncontractile tails (c2-like phages; Jarvis *et al.*, 1991). The P335 and BK5-T species contain both lytic and temperate phages, in contrast to the c2 species (recently assigned genus status, Maniloff and Ackermann, 1998), which only contains lytic phages. Generally, prolate-headed phages are encountered less frequently than isometric-headed phages. It was reported that 85% of the virulent lactococcal phages isolated in New Zealand have small isometric heads, with most of the remainder having a prolate head, and the balance having large isometric heads (Jarvis, 1977; Terzaghi, 1976). Prolate phages are particularly significant because they have a wider host range than small isometric-headed phages and therefore they can infect a greater variety of starter strains (Heap and Jarvis, 1980).

1.2.3 Lactococcal phage life cycle

Lactococcal phage, and most other bacteriophage, have a sequential life cycle that involves several key steps. The sequence of events is: adsorption of phage to the

host cell followed by DNA entry, early gene expression, DNA replication, late gene expression, phage particle assembly, and finally release of progeny phage by host cell lysis.

Lactococci are Gram-positive bacteria and have an external cell wall that is much thicker than that of Gram-negative bacteria such as *E. coli*. Therefore, phages of lactococci and other Gram-positive bacteria generally adsorb to the cell wall prior to interaction with the membrane. In most cases, lactococcal phages attach via their tails to a carbohydrate moiety of the cell wall. The interaction, which is usually via a galactose and/or rhamnose residue (Valyasevi *et al.*, 1990; Monteville *et al.*, 1994), is initially reversible, but becomes irreversible over time as the infection continues. In the next step, penetration of the host membrane, the phage appears to interact with a specific membrane protein. In *L. lactis*, this phage infection protein (Pip) is essential for phage c2 infection, as Pip deficient strains of *L. lactis* ssp. *lactis* C2 fail to irreversibly adsorb phage c2 (Geller *et al.*, 1993). Another protein (32 kDa) with the ability to bind phage has been purified from the membrane fraction of *L. lactis* ssp. *lactis* C2 (Valyasevi *et al.*, 1991).

In a lytic phage infection, in the absence of any resistance mechanisms, shut-down of the host metabolism and initiation of phage DNA replication is mediated by the early phage genes. Currently, little is known about lactococcal phage DNA replication. In phage c6A replication, the host cell DNA is degraded by phage nucleases and the products incorporated into the phage DNA (Powell *et al.*, 1992). In the temperate lactococcal phage Tuc2009, a DNA replication module has been identified which contains genes encoding putative single-stranded DNA binding proteins, a topoisomerase I, a methylase, and a replisome organizer protein. The putative replisome organizer protein (Rep_{2009}) has been shown in gel retardation assays to bind to ori_{2009} (McGrath *et al.*, 1999). In another temperate phage, TP901-1, two proteins have been identified which are essential for *in vivo* phage DNA replication; a single-stranded DNA binding protein and a putative replication initiation protein (Østergaard *et al.*, 2001). In the temperate P335 phage r1t, a putative replisome organizer protein has also been identified. This protein (Pro11) acts as a functional analog of DnaA and shows high homology with Rep_{2009} (from phage Tuc2009) and G38P (from *Bacillus subtilis* phage

SSP1). Pro11 has been shown to be a DNA-binding protein that specifically binds to a set of direct repeats (Zúñiga *et al.*, 2002).

The cloned sk1 (936-type phage) origin of replication can support plasmid replication in *L. lactis* if the N-terminal 179 codons of ORF (open reading frame) 47 and the intergenic region between ORFs 47 and 48 are present on a plasmid which does not contain a *L. lactis* origin of replication (Chandry *et al.*, 1997). The cloned c2 *ori* can also support plasmid replication in *L. lactis* in the absence of phage-encoded proteins (Waterfield *et al.*, 1996).

After expression of the phage structural genes, the phages are assembled. To date, several structural proteins of lactococcal phages have been identified (see section 1.2.7). After phage assembly, the phage DNA is packaged and the phages are released by cell lysis, a process catalyzed by the cell wall degrading enzyme, lysin. Phage endolysins are diverse and show different muralytic activities (glycosylase, amidase, endopeptidase and transglycosylase; Young *et al.*, 2000). However, in Gram-negative and Gram-positive bacteria, cell lysis by most bacteriophages requires the presence of a second lysis component, holin. Holin causes non-specific lesions in the membrane, which allow access of lysin to its murein substrate. Therefore, holin expression controls the timing of lysis (Young *et al.*, 2000; for review see Wang *et al.*, 2000).

In addition to lytic lactococcal phages, there are also temperate lactococcal phages (e.g. BK5-T, TPW22, r1t and TP901-1), which integrate their genomes into the host chromosome by site-specific recombination to establish a lysogenic life cycle (Boyce *et al.*, 1995; Petersen *et al.*, 1999, van Sinderen *et al.*, 1996).

1.2.4 Bacterial resistance to lactococcal phage attack

Lactococci have developed several mechanisms for preventing phage attack that disrupt the phage life cycle at various stages (for review see Coffey and Ross, 2002; Garvey *et al.*, 1995a). These mechanisms include: adsorption inhibition, prevention of phage DNA injection, restriction and modification systems (R/M), and abortive infection mechanisms (Abi's, for review see Forde and Fitzgerald, 1999).

Adsorption inhibition can occur in two ways: the phage receptor may be absent from the cell surface, or it can be masked by exopolysaccharides that are produced by the resistant cell (Sijtsma *et al.*, 1988, 1990). Host mediated systems that inhibit phage DNA injection have also been reported (Garvey *et al.*, 1996), for example DNA penetration can be blocked by mutation of an uptake protein (Valyasevi *et al.*, 1991; Kraus and Geller, 2001). Phages may hinder superinfection by blocking a DNA injection step of an infecting phage. An example is the Sie_{2009} protein of Tuc2009 (P335 phage), which is associated with the cell membrane and allows phage adsorption, but not DNA entry of phages of the 936 species (McGrath *et al.*, 2002).

Many bacteria have developed strategies to recognize phage DNA and to cleave it after successful entry of the phage genome into the host cell using restriction endonucleases (Schouler *et al.*, 1998a; O'Sullivan *et al.*, 2001; for review see Forde and Fitzgerald, 1999; Coffey and Ross, 2002). Bacteria that utilize such a defense system protect their own genome from auto-degradation by self-methylation of their DNA so that the restriction endonuclease can no longer act. Obviously such a system must be tightly regulated to prevent auto-restriction and death of the host cell. Some bacteriophages have adapted to this protective mechanism. Phage ϕ50 for example, contains a functional part of the methylase *Lla*I and is therefore capable of methylating its own genome in any propagating host, and thus eluding restriction by certain restriction enzymes (Hill *et al.*, 1991).

Phage resistance mechanisms, that interfere with intracellular phage development after phage DNA has entered the host intact, are called abortive infection mechanisms (Abi) and they can affect the phage life cycle at various stages: Abis can take place at the level of phage genome replication, transcription/translation, phage DNA packing, assembly, and cell lysis and phage release (see Table 1.3). Generally, the Abi-mediated resistance kills the host either through the action of the Abi protein itself or because lethal alterations of the host functions have been initiated by the phage infection. However, the death of a single cell is insignificant as long as it prevents the phages from multiplying and infecting other cells.

Table 1.3. Lactococcal abortive mechanisms.

Abi	Phage affected	Level at which Abi is acting	Reference
AbiA	936 sp. (sk1), P335 sp. (31), c2 sp. (c2)	phage DNA replication	Hill *et al.* (1990a); Coffey *et al.* (1991); Dinsmore and Klaenhammer (1994, 1997); O'Sullivan *et al.* (1995); Dinsmore *et al.* (1998)
AbiB	936 sp. (bIL66, bIL70)	phage transcription	Cluzel *et al.* (1991); Parreira *et al.* (1996); Forde *et al.* (1999)
AbiC	936 sp. (p2, jj50, sk1), P335 sp. (ul136, ml2r)	major capsid protein	Durmaz *et al.* (1992)
AbiD	936 sp. (sk1), c2 sp. (c2)	after phage DNA replication	McLandsborough *et al.* (1995)
AbiD1	936 sp. (bIL66, bIL70), c2 sp. (c6A, bIL67)	major capsid protein	Anba *et al.* (1995); Bidnenko *et al.* (1995)
AbiE	936 sp. (712), c2 sp.	?	Garvey *et al.* (1995b)
AbiF	936 sp. (712), c2 sp. (c2)	phage DNA replication	Jarvis (1988, 1992); Garvey *et al.* (1995b)
AbiG	936 sp. (712, sk1), c2 sp. (c2)	phage transcription	O'Connor *et al.* (1996, 1999)
AbiH	936 sp. (59), c2 sp. (53)	?	Prévots *et al.* (1996)
AbiI	936 sp. (712), c2 sp. (c2)	phage DNA packaging	Su *et al.* (1997)
AbiJ	936 sp. (712)	?	Deng *et al.* (1997)
AbiK	936 sp., c2 sp., P335 sp.	phage DNA replication	Émond *et al.* (1997); Boucher *et al.* (2000, 2001)
AbiL	936 sp. (712), c2 sp. (c2)	after transcription	Deng *et al.* (1999)
AbiN	936 sp. (59), c2 sp. (53)	?	Prévots *et al.* (1998)
AbiO	936 sp. (59), c2 sp. (53)	?	Prévots and Ritzenthaler (1998)
AbiP	936 sp	?	Schouler *et al.* (1998b)
AbiQ	936 sp. (p2), c2-like sp. (p2)	accumulation of replicative form of DNA	Émond *et al.* (1998), Boucher *et al.* (2001)
AbiR	936 sp, c2 sp.	phage DNA replication	Twomey *et al.* (2000)
AbiS	936 sp.	phage development	Matvienko *et al.* (1999)
AbiT	936 sp., P335 sp.	?	Bouchard *et al.* (2000, 2002)
AbiU	936 sp. (712), c2 sp. (c2), P335 sp. (ul36)	phage transcription	Dai *et al.* (2001)

Table modified from Forde *et al.* (1999).

Abi systems are characterized by a reduction in the EOP (efficiency of plaquing), a decrease in the ECOI (efficiency of the centre of infection) and the development of smaller plaques due to a decrease in burst size. Most Abi systems are plasmid-encoded and have a [G+C] content of approximately 26-29% (Hill *et al.*, 1990a; Cluzel *et al.*, 1991; Durmaz *et al.*, 1992), which is in contrast with the

average value of approximately 38% [G+C] for lactococcal genes. The low [G+C] content has been hypothesized to be due to horizontal gene transfer from other species of low [G+C] content (O'Connor *et al.*, 1996). So far 21 Abis have been identified with the mode of action of most of them being only partially understood (Table 1.3).

1.2.5 Strategies to reduce infection of dairy starter cultures with bacteriophages

Strategies based on natural and genetically engineered phage resistance mechanisms have successfully prevented phage infection of industrially important lactococcal strains (Batt *et al.*, 1995). Mechanisms include those that disrupt either phage adsorption, DNA entry, phage DNA replication, or the expression of phage structural proteins (for review see Daly *et al.*, 1996; Coffey and Ross, 2002). There are several examples of genetically engineered phage resistance mechanisms in the literature.

Resistance against phage c2 infection has been achieved by replacing a gene for a bacteriophage receptor (*pip*) in the *L. lactis* host with a mutated allele (Garbutt *et al.*, 1997). In another approach, phage DNA replication has been prevented by introducing a restriction and modification system into the lactococcal starter strain (Madsen and Josephsen, 1998).

Antisense mRNA, the transcription product of the DNA strand complementary to the coding strand, has also been used to generate phage resistant strains. In this method, formation of a sense:antisense duplex either blocks expression of phage genes by interfering with ribosome binding and translation (Andersen *et al.*, 1989) or increases the rate of RNA degradation (for review see Braasch and Corey, 2002).

This technology can be targeted against both specific and conserved bacteriophage gene functions (for review see Kim *et al.*, 1992). One of the earlier examples using antisense RNA to generate phage resistance was reported by Chung *et al.* in 1992, who discovered that an antisense RNA construct, directed against the mRNA coding for the major capsid protein (MCP) of phage F4-1, could be used to inhibit phage infection. However, because the major capsid proteins are produced in excess, this construct only provides a modest level of resistance against phage infection. In contrast, good protection against ϕ31 phage infection in *L. lactis* can be obtained by

introducing a low-copy number vector containing the ϕ31 *ori* and different antisense ORFs into the bacteria. In this system, the presence of *ori* 31 results in explosive amplification of the low-copy number vector upon phage infection (Walker and Klaenhammer, 2000) and therefore increases production of antisense RNA. The possibility of using antisense RNA against bacteriophage attack has also been demonstrated in *Streptococcus thermophilus* (Sturino and Klaenhammer, 2002), a lactic acid bacterium frequently used in the production of yoghurt.

A triggered suicide system has also been developed. In this system, a phage-inducible promoter (from ϕ31, Walker and Klaenhammer, 1998) fused to the restriction cassette from the *LlaI* R/M system (Djordjevic *et al.*, 1997) results in abortion of phage infection by intentionally killing the propagating host through the action of the restriction endonuclease (Djordjevic and Klaenhammer, 1997a).

Another approach to prevent phage lysis of *L. lactis* involves the use of a neutralizing heavy-chain antibody fragment from llamas. This antibody fragment recognizes a phage structural protein located at the base plate of phage P2 (936-type phage) which is involved in host recognition (Ledeboer *et al.*, 2002). Thus antibody binding prevents phage adsorption to the host.

Another engineered resistance mechanism that prevents phage replication has been developed against small isometric phages of the P335 species. In this system, the *ori* of DNA replication is present in *trans* on a high copy number plasmid (ϕ31, O'Sullivan *et al.,* 1993; ϕ50, Hill *et al.*, 1990b; TP901-1, Østergaard *et al.*, 2001; Tuc2009, Q30, Q33 and ul36, McGrath *et al.*, 2001; and BK5-T, Mahanivong *et al.*, 2001). When host cells carrying phage *oris* cloned into a plasmid, are infected by the corresponding phage, an increase in plasmid replication is observed as well as a decrease in phage propagation. This Per phenotype (phage encoded resistance) is presumed to be caused by the titration of proteins essential for phage DNA replication. The Per phenotype is not unique to lactococcal phages, and has also been observed in the *Lactobacillus casei* phage A2 (Moscoso and Suárez, 2000) and in the *Streptococcus thermophilus* phage Sfi21 (Foley *et al.*, 1998).

However, there are not only technical limitations to the current ability to produce phage resistant bacterial strains, but there is also great public concern in

genetically modifying bacteria that are used in food production for human consumption (Kondo and Johansen, 2002). In addition, phage can overcome particular phage-resistance mechanisms by acquiring non-specific point mutations in their genome, or by uptaking DNA from the host or from co-infecting phages or prophages (Coffey and Ross, 2002; Bouchard and Moineau, 2000; Durmaz and Klaenhammer, 2000). Therefore a better understanding of phage biology is required to fight phage infection of industrially important lactococcal strains.

1.2.6 Genome size and structure of lactococcal phages

To date all lactococcal phages investigated have a double-stranded, linear DNA genome with a [G+C] content similar to their hosts (35-41%, Jarvis, 1989). The majority have cohesive ends which consist of single-stranded 3' overhangs of variable length (Chandry *et al.*, 1994; Lillehaug *et al.*, 1991; Lubbers *et al.*, 1994). Generally the phage genomes have only small non-coding regions (Schouler *et al.*, 1994) and range from 29 to 40 kb for small isometric phages, 52 to 134 kb for large isometric phages and 18 to 22 kb for prolate-headed phages (Jarvis *et al.*, 1991; Prévots *et al.*, 1990). Restriction maps have been constructed for several phages including c2, sk1 (Pillidge and Jarvis, 1988), c6A (Powell *et al.*, 1989), BK5-T (Lakshmidevi *et al.*, 1988), 853, 936, and 1374 (Jarvis and Klaenhammer, 1986). Restriction analysis of lactococcal phage DNA has shown that there are fewer restriction sites than normally expected, and in particular for enzymes with 4-base recognition sequences (Pillidge and Jarvis, 1988; Powell and Davidson, 1986; Relano *et al.,* 1987). The estimate of the total number of restriction sites in genomes was calculated from the genome length and [G+C] content. Elimination of restriction enzyme sites could be a mechanism, which allows the phage to escape restriction by the host.

To date several lactococcal phages have been completely sequenced: the genomes of small isometric 936 group phages sk1 (Chandry *et al.*, 1997) and bIL170 (Crutz-Le Coq *et al.*, 2002), the small isometric group P335 phages r1t (van Sinderen *et al.*, 1996), TP901-1 (Brøndsted *et al.*, 2001), Tuc2009 (GenBank accession AF109874), bIL285, bIL286, bIL309 (Chopin *et al.*, 2001), and ul36 (Labrie and Moineau, 2002),

BK5-T (Boyce *et al.*, 1995; Mahanivong *et al.*, 2001) and the prolate-headed phages c2 (Lubbers *et al.*, 1995) and bIL67 (Schouler *et al.*, 1994).

With the availability of several complete phage genomes, comparative phage genomics using bioinformatics tools has become possible (Brüssow and Desiere, 2001; Desiere *et al.*, 2001; Brüssow and Hendrix, 2002; Desiere *et al.*, 2002).

1.2.7 Genes with identified functions

A large number of lactococcal phage genes have been functionally characterized (for review see Djordjevic and Klaenhammer, 1997b; Brüssow, 2001). The first gene cloned from a lactococcal phage was the lysin gene of the prolate phage ϕvML3 (Shearman *et al.*, 1989). Several more lysin genes have since been identified, cloned and experimentally characterized (ϕUS3, Platteeuw and de Vos, 1992; c2, Ward *et al.*, 1993; Tuc2009, Arendt *et al.*, 1994, reviewed in Gasson, 1996). Additional genes have been identified encoding holins (Tuc2009, Arendt *et al.*, 1994; c2, Lubbers *et al.*, 1995) and a transcriptional repressor (Tuc2009, van de Guchte *et al.*, 1994). Also putative genes encoding structural proteins have been identified (Tuc2009, Arendt *et al.*, 1994; F4-1, Chung *et al.*, 1991) and experimentally characterized (c2, Lubbers *et al.*, 1995; ϕ197, Schouler *et al.*, 1992; TP901-1; Pedersen *et al.*, 2000). Genes encoding a putative regulator (BK5-T, Lakshmidevi *et al.*, 1990), an endonuclease (bIL66, Bidnenko *et al.*, 1998), a replication protein (TP901-1, Østergaard *et al.*, 2001), a transcriptional repressor (r1t, Nauta *et al.*, 1996) and a lysis/lysogeny regulatory protein (TP901-1, Madsen et al., 1999; ϕLC3, Blatny *et al.*, 2001) have been experimentally characterized. Sequence analysis identified genes encoding integrases (BK5-T, Boyce *et al.*, 1995; r1t, van Sinderen *et al.*, 1996) and some have been experimentally confirmed (TP901-1, Breüner *et al.*, 2001; TPW22, Petersen *et al.*, 1999; ϕLC3, Lillehaug and Birkeland, 1993).

1.3 The lactococcal phage c2

The lytic lactococcal phage c2 belongs to the *Siphoviridae* family (morphotype B2) and is characterized by a prolate head (50 x 36 nm) and a long noncontractile tail (94 nm long) (Figure 1.2).

Figure 1.2. Electronmicrograph of phage c2.
The bar represents the scale of the electronmicrograph (courtesy of J. Rakonjac).

1.3.1 The phage c2 genome

Analysis of the fully sequenced c2 genome (GenBank accession number L48605; Lubbers *et al.*, 1995; Jarvis *et al.*, 1995) shows it to have 80% homology at the nucleic acid level with phage bIL67 (Lubbers *et al.*, 1995) and a [G+C] content of 36%. The 22,163 bp linear double-stranded DNA genome contains cohesive ends (*cos* ends), which consist of nine nucleotide single-stranded 3' overhangs (5'-GTTAGGCTT-3', 3'-CAATCCGAA-5', Lubbers *et al.*, 1994). The *cos* site contains a TCAN indirect repeat that is highly conserved in c2 species of lactococcal bacteriophages (Lubbers *et al.*, 1994; Lubbers *et al.*, 1995; Perrin *et al.*, 1997) and may be related to terminase specificity.

The genomes of most phages circularize after injection into the bacterial cytoplasm (reviewed in Murialdo, 1991) if their ends are not protected by a protein. In phage c2, base pairing of the complementary single-stranded ends occurs (i. e. circularization) after injection of the phage DNA into the cytoplasm of the host cell, followed by lysis of the host approximately 30 min after initiation of infection (Lubbers *et al.*, 1994).

Phage c2 early and late genes have been identified by sequence (Lubbers *et al.*, 1995) and transcription analysis (Beresford *et al.*, 1993; Lubbers *et al.*, 1998). The early and late genes are organized into two divergently oriented blocks that are separated by a 611 bp noncoding (intergenic) region containing the origin of DNA replication (Lubbers *et al.*, 1995; Waterfield *et al.*, 1996). The early region of the genome contains six leftward-directed promoters (P_E1-P_E6), and the late region has a single rightward-oriented promoter (P_L1). The early promoters P_E4, P_E5 and P_E6 have perfect -35 and -10 consensus promoter hexamers separated by 17 bp, compared to the consensus recognition sequence for the *L. lactis* σ^{39} transcription factor (TTGACA...17bp...TATAAT...6bp). In contrast, the early promoters P_E1 and P_E2 contain deviations from the consensus -35 sequence while P_E3 has perfect -10 and -35 sequences but they are separated by 20 bp instead of 17 bp. Finally, the late promoter P_L1 has a perfect -10 sequence but no apparent -35 sequence (Lubbers *et al.*, 1998). Thus most of the promoters show a perfect or nearly perfect *L. lactis* σ^{39} consensus promoter sequence. This combined with the fact that detailed c2 genome sequence analysis has shown no evidence for a phage encoded RNA polymerase strongly suggests that c2 uses the RNA polymerase encoded by the host. Chloramphenicol treatment prior to RNA isolation from lactococcal cells infected with c2 resulted in a decrease in late transcript levels. This indicates that a phage protein may activate P_L1, or the decrease could be due to competition between early and late promoters for the transcription apparatus (Lubbers *et al.*, 1998).

Transcription of the early genes starts within two minutes, and transcription of the late genes within four to six minutes after initiation of infection (Lubbers *et al.*, 1998). While transcription initiation of the early and late transcripts corresponds to the predicted start sites, P_E3 has been shown to be non-functional as a promoter,

presumably due to its divergence from the consensus promoter sequence (Lubbers *et al.*, 1998).

In the phage c2 genome, only 1.5 kb (7%) of noncoding sequences have been found (Lubbers *et al.*, 1995) and a total of 22 open reading frames (ORFs) have been predicted for the early region and 17 for the late region (Figure 1.3). This prediction was based on the following criteria: an ORF must start with an AUG, GUG, UUG or AUA start codon, it must encode a product greater than 20 amino acids, and must be either preceded by a Shine-Dalgarno sequence or have the potential for translational coupling. To date, putative functions have been assigned to 12 genes using sequence analysis and/or experimental evidence (Lubbers *et al.*, 1995).

In the early region, sequence analysis revealed some similarity between an ORF (e22) and a functional domain of a sigma-factor (Lubbers *et al.*, 1998). An ORF (e12) encoding a putative helix-turn-helix (HTH) containing protein has also been identified; data suggest that this protein may bind to a specific DNA sequence and function as a transcription regulator. A putative recombination protein (ORF e15) has been identified by virtue of its high homology with recombination proteins of two *Salmonella* phages (Lubbers *et al.*, 1995).

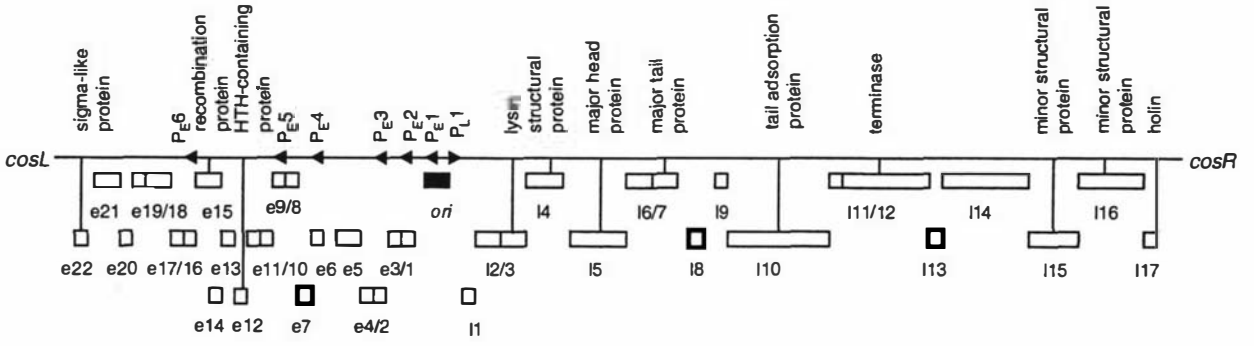


Figure 1.3. Schematic diagram of the c2 genome with putative and known gene functions.

The rectangles represent ORFs of the early and late region of the c2 genome, the black arrows indicate promoters (P_E1-P_E6, and P_L1) and their orientation, and the black box represents the origin of replication. Figure reproduced and modified from Lubbers *et al.* (1995).

In the late region, a putative holin protein (ORF 117), a lysin (ORF 13), several structural proteins (ORFs 14, 15, 17, 115 and 116) and a putative terminase (ORF 112; which may be responsible for cleaving and packaging the phage DNA), have been identified (Lubbers *et al.*, 1995; Ward *et al.*, 1993). The lysin gene has been cloned and expressed in *E. coli* and its function has been experimentally confirmed (Ward *et al.*, 1993), while immunogold labelled antibodies have shown that ORF 15 encodes a major head protein and ORF 17 encodes a major tail protein (Lubbers *et al.*, 1995).

A single termination site is located downstream of the last ORF e22 (early gene region, Lubbers *et al.*, 1995). This transcription termination sequence can form a stem structure and is conserved among members of the c2 group (Perrin *et al.*, 1997); no termination signal is found downstream of the last late gene.

1.3.2 The c2 origin of DNA replication

The c2 origin was first identified within a non-coding region by sequence analysis of the c2 genome (Lubbers *et al.*, 1995). The 611 bp noncoding region that separates the early genes from the late genes, contains a highly AT-rich region (78%, bases 7010-7231 in the c2 genome) and consists of several small perfect and imperfect inverted and direct repeats. This part of the genome is also highly conserved in all other prolate lactococcal phages examined to date, in contrast to the poorly conserved region downstream of P_E1 (Waterfield *et al.*, 1996; Rakonjac *et al.*, submitted). Prokaryotic replication origins are known to contain AT-rich repeats, although these repeats differ between origins (for review see Bramhill and Kornberg, 1988). It is in these AT-rich regions where strand opening and assembly of host initiation factors occur. A dramatic change in the polarity of GC skew was also found around this non-coding region (Callanan *et al.*, 2001), which is consistent with the presence of an origin of bi-directional DNA replication (McLean *et al.*, 1998).

The 611 bp noncoding sequence also contains a leftward oriented promoter (P_E1; Figure 1.4). Construction of a detailed transcription map of phage c2 (Lubbers *et al.*, 1998) has shown that there are three transcripts synthesized (approximately 260 nt, 300 nt and 360 nt) from P_E1 (Figure 1.4). Sequence analysis of the P_E1 transcripts suggests that they are not translated (using the criteria for the sequence analysis of

ORFs listed on page 17). The level of transcripts synthesized increases during the course of c2 infection but the role of the transcripts in phage c2 replication remains unclear.

When a 521 bp DNA fragment containing the early promoter 1 (P_E1) and the late promoter 1 (P_L1) was cloned into the origin screening vector pVA891, it supported plasmid replication in *L. lactis* in the absence of phage proteins (Waterfield *et al.*, 1996). The plasmid pVA891 has an origin of replication functional in Gram-negative (*E. coli*), but not in Gram-positive bacteria (Macrina *et al.*, 1983). From this it can be concluded that the cloned c2 DNA fragment contains a c2 origin of replication. However, a 260 bp fragment consisting of P_E1 and P_L1, but not the region downstream of P_E1, is not capable of supporting plasmid replication (Waterfield *et al.*, 1996), thus the region downstream of P_E1 must also be essential for plasmid replication.

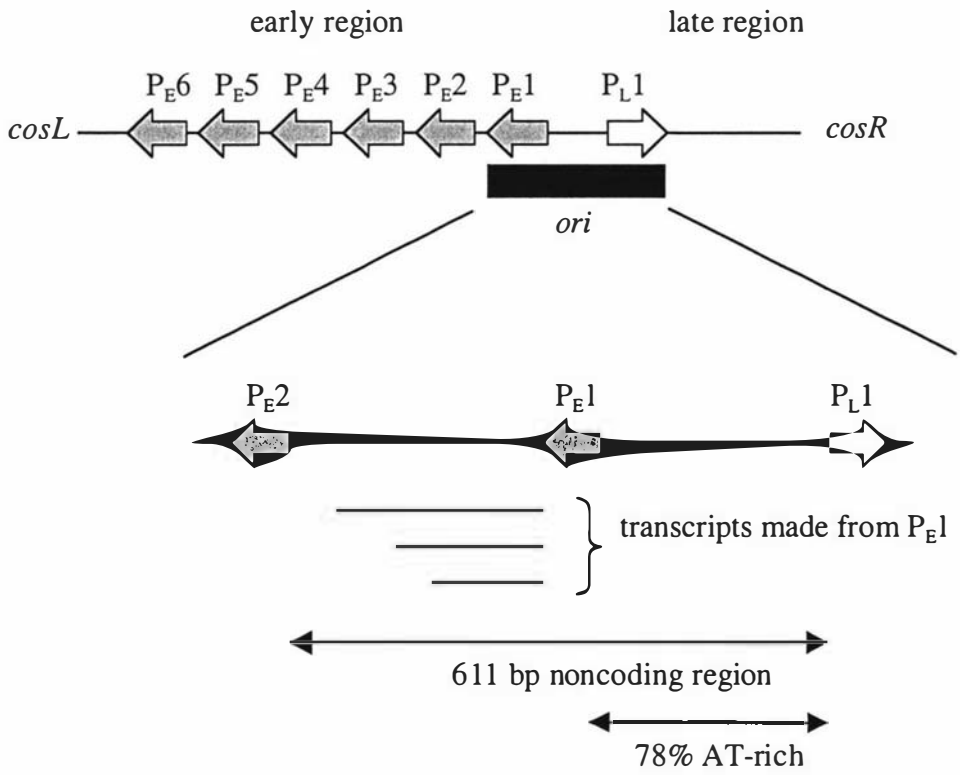


Figure 1.4. Schematic diagram of the c2 origin of replication.

The gray arrows represent the early promoters, the white arrow the late promoter, and the c2 origin is shown as a black box. The transcripts synthesized from P_E1 are drawn as thin black lines, and the noncoding region and the highly AT-rich region are both represented by arrows. Figure not drawn to scale.

A cloned phage origin would be expected to confer a Per (phage encoded resistance) phenotype if it bound phage-encoded proteins essential for replication. The presence of the cloned c2 origin of replication does not inhibit c2 replication efficiency (Polzin *et al.*, 1999) and therefore phage c2 does not show a Per phenotype. This suggests that phage c2 may use a distinct mechanism for DNA replication which is dependent exclusively on host-encoded proteins. This is in contrast to many lactococcal phages that confer a Per phenotype (see section 1.2.5).

Recently it has been demonstrated by two-dimensional gel electrophoresis that phage c2 replicates via theta replication and that the *ori*-locus encodes the origin of theta type replication (Callanan *et al.*, 2001). However, some data produced by two-dimensional gel electrophoresis cannot be interpreted easily. These data may point to another origin of replication utilized by c2 or another mechanism of replication, in addition to theta replication initiating from the *ori* locus (Callanan *et al.*, 2001). It is not unusual for phages to employ more than one mechanism of DNA replication: for example in coliphage λ DNA replication, most molecules switch to rolling-circle replication after a period of theta replication (reviewed in Campbell, 1994).

1.4 DNA replication in prokaryotes

Bacteria have circular chromosomes that contain one or several origins of replication. In *E. coli* chromosomal replication starts from a single origin termed *oriC* and proceeds bidirectionally from this point. The rate at which the DNA polymerases move along the *E. coli* chromosome is approximately 100 kb per minute; thus the whole chromosome can be replicated from a single origin (*oriC*) in 40 minutes (Kornberg and Baker, 1992).

Genome replication occurs in several phases: initiation, elongation and termination (Kornberg and Baker, 1992). The process of initiation can be divided into multiple steps. In the first step, initiator proteins recognize specific DNA sequences within the genome. This interaction determines the location of the origin of replication. The pre-replication protein complex then renders the genome competent for replication (e.g. unwinding of the origin). The DNA helicase (unwinds the DNA further), the

primase (provides primers for initiation of DNA synthesis) and the DNA polymerase III holoenzyme have to be attracted to the origin and form a multienzyme complex which is called the replisome. DNA synthesis starts and proceeds over the whole genome (elongation) until termination of replication occurs at a distinct region of the genome and the daughter chromosomes separate (reviewed in Benkovic *et al.*, 2001).

1.4.1 Structural elements involved in replication

Fine-structure analysis of origins of replication from enteric bacteria by evolutionary comparison and mutagenesis has revealed that the bacterial chromosomal replication origin is highly conserved between species and is composed of several essential DNA sequence elements (Moriya *et al.*, 1999; Ogasawara *et al.*, 1990).

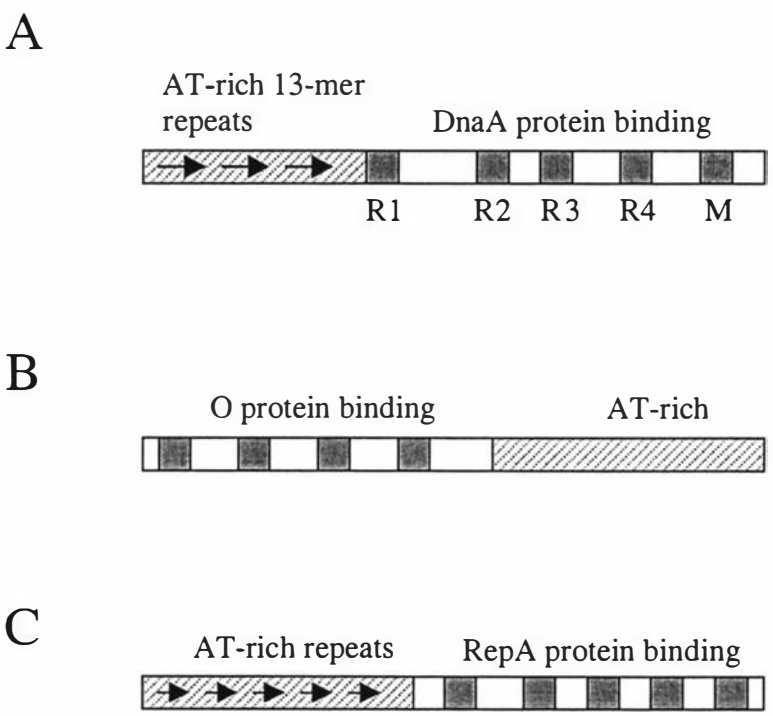


Figure 1.5. Structural elements of replication origins.

(A) *oriC* of *E. coli*, (B) *oriλ* of phage λ, and (C) *oriP1* of plasmid P1. The gray boxes represent repeats to which DNA binding proteins bind, the shaded boxes represent AT-rich sequences, and arrows indicate repeats. Figure reproduced and modified from Weigel *et al.* (2001), Marians (1992), Szalewska-Palasz *et al.* (1998) and Chattoraj (2000).

One of these sequence elements consists of multiple, short repeated sequences that are important for replication protein binding. Another important element is a highly AT-rich sequence that usually contains repeats. These AT-rich sequences, called DNA unwinding elements (DUEs) are thermodynamically unstable and so facilitate opening of the strands (reviewed in Bramhill and Kornberg, 1988).

One example of the replication protein-binding repeats are the DnaA boxes. Bacterial origins contain different numbers of DnaA protein (initiator protein) binding sites (DnaA boxes). In *E. coli* there are five DnaA boxes (9-mer; R1, R2, R3, R4, and M; Weigel *et al.*, 2001), and there are 15 in *Bacillus subtilis*. Binding of DnaA protein to the DnaA boxes initiates replication, recruits more replication proteins to *oriC*, and unwinds three 13-mer repeats within the AT-rich sequence in *oriC* of *E. coli* (Figure 1.5).

Instead of, or in addition to the DnaA boxes, phages and plasmids contain different types of repeats that allow binding of the initiation protein to DNA in the origin region (Figure 1.5). In most cases the origins of phages and plasmids also contain an AT-rich region (e. g. λ, T7 and P1).

1.4.2 The *E. coli* chromosome as a model system for DNA replication

DNA replication of the chromosome in *E. coli* starts from *oriC* and several copies of the DnaA protein bind to the DnaA boxes (for review see Messer and Weigel, 1997). In the presence of ATP, the three tandem 13-mer AT-rich repeats melt to form an open complex (Speck and Messer, 2001).

Open complex formation is aided by either HU protein (histone-like protein) and/or IHF (integration host factor), and the SSB protein (single-stranded DNA binding protein) stabilizes the complex. The replicative helicase (DnaB protein) assembles with the help of DnaC protein (helicase loading factor) on the origin to form the prepriming complex, followed by assembly of the primosome complex (which consists of PriA (helicase), PriB, PriC, DnaT, DnaC and DnaB; Marians, 1992). DnaC is then released and the helicase unwinds the origin further allowing the DnaG protein (primase, for review see Frick and Richardson, 2001) to enter and synthesize the necessary RNA

primers. Finally the replicative polymerase enters and DNA synthesis is initiated (Fang *et al.*, 1999; Table 1.4).

Table 1.4. Major proteins involved in replication of *E. coli*.

Name	Function
DnaA	ATP-binding protein, binds to origin, initiates replication
DnaB	DNA helicase, unwinds DNA
DnaC	loading factor for helicase
DnaG	DNA primase
Gyrase A	subunit A of DNA gyrase
Gyrase B	subunit B of DNA gyrase
DNA ligase	DNA ligase
PolA	DNA polymerase I (repair enzyme), also removal of RNA primer
Pol III	DNA polymerase III, major enzyme for DNA synthesis (see Table 1.5 for details)
NrdAB	Ribonucleotide reductase, synthesizes deoxyribonucleotides from ribonucleotides
RpoA	α subunit of RNA polymerase
RpoB	β subunit of RNA polymerase
RpoD	σ subunit of RNA polymerase, responsible for promoter binding
SSB	single-stranded DNA binding protein, facilitates melting of helices and stabilizes single strands

Table modified from Birge (2000).

The polymerase III holoenzyme consists of 10 unique subunits (α, ε, θ, τ, γ, δ, δ', χ, ψ, and β; Table 1.5). The catalytic Pol III core is composed of a heterotrimer of α, ε and θ (Table 1.5) and contains the DNA polymerase activity and the proofreading 3'-5' exonuclease. This Pol III core enzyme has very low processivity. Binding of τ to the Pol III core causes the enzyme complex to dimerize, generating Pol III' and processivity of the enzyme increases several-fold with this step. Binding of a γδ complex (consisting of γ, δ, δ', χ, ψ) to Pol III' generates Pol III* and addition of the β subunit generates the functional Pol III holoenzyme (for review see Sutton and Walker, 2001).

The replisome complex is stationary during replication and the DNA moves through it (Lemon and Grossman, 1998; for review see Cook, 1999).

DNA replication in *E. coli* stops at the Ter-sites (replication termini) in a region of the genome that is 180° from the origin. The Tus protein binds to the Ter-sites and functions as an anti-helicase complex. Recent data suggest that the Tus protein interacts with the DnaB protein and thereby inhibits DnaB activity (Mulugu *et al.*, 2001).

Table 1.5. Composition of DNA polymerase III holoenzyme.

Subunit	Function	Complex
α	DNA polymerase	Pol III core
ε	3'-5' exonuclease	
θ	stimulates exonuclease	
τ	stimulates helicase, dimerizes core	Pol III"
γ	ATP-requiring clamp loader	Pol III*
δ	binds to β	
δ'	stimulates ATPase of γ	
χ	binds SSB	
ψ	accessory protein	
β	clamp protein	Pol III holoenzyme

Table modified from Benkovic *et al.* (2001); Baker and Bell (1998)

For lagging strand replication, the RNA primers are removed either by RNase H or the exonuclease activity of DNA polymerase I, followed by joining of the Okazaki fragments by DNA ligase. During replication, extra twists are introduced in the unreplicated DNA and must be removed by topoisomerase I (for review see Postow *et al.*, 2001), while in the newly replicated DNA helices the normal supercoiled state is restored by DNA gyrase (for review see Benkovic *et al.*, 2001).

GATC sequences in the origin are normally methylated at the adenyl residue by dam methylase. However, after replication these sites are hemimethylated. New initiation from *oriC* is only possible if the sites are fully methylated (this takes approximately 10 min; Landoulsi *et al.*, 1989; for review see Katayama, 2001). Recent data suggest that replication is also regulated by the Hda (homologous to DnaA) protein which inactivates DnaA (Kato and Katayama, 2001).

1.4.3 Transcriptional elements as components of origins

An obligatory step in replication in some organisms is the transcription by RNA polymerase of origin DNA sequences into short RNA molecules. There are two possible explanations why transcription is required for replication: either transcription creates RNA molecules that can then be processed by RNA processing enzymes (for example

RNase H) to serve as a primer, or transcription opens the double helix to allow binding of a primosome which then synthesizes the actual primer (Figure 1.6, transcriptional activation; Hassan and Cook, 1994). It has been suggested that the involvement of RNA polymerase in DNA replication is a relic of an RNA world (Matsumoto, 1994).

The involvement of RNA polymerase in *E. coli* replication was discovered by the observation that initiation of replication is rifampicin sensitive, independent from the requirements for protein synthesis (Lark, 1972, rifampicin inhibits transcription initiation). In *E. coli*, transcription by RNA polymerase from a strong promoter (*mioC*) adjacent to *oriC* separates the two DNA strands of the origin so that specific sequences are exposed to the primosome (transcriptional activation, Baker and Kornberg, 1988). Several experiments support this model: *E. coli* RNA polymerase can be replaced by T7 or T3 polymerases when *oriC* is cloned into a plasmid containing promoters for these polymerases; RNA terminated with 3' dATP (lacking a 3' OH group) still activates initiation of replication; and the RNA is effective 200 bp from *oriC*, and can be synthesized from either side of *oriC* (Baker and Kornberg, 1988). All the above data suggest that transcription itself activates replication rather than providing a primer for replication.

Transcription initiated from p_R in phage λ proceeds into the λ replication region (Taylor and Wegrzyn, 1995). Deletion of p_R in a plasmid system reduces the efficiency of the cloned λ origin to support replication of the plasmid, but can be restored by inserting a *lac* promoter into *oriλ*. Furthermore, transcription downstream of *oriλ* is necessary for replication, but the sequence is irrelevant. All these results suggest that transcription by RNA polymerase serves in transcriptional activation of *oriλ*, and that the transcript synthesized from p_R does not serve as a primer (Hase *et al.*, 1989). The *E. coli* DnaA protein directly activates the λ p_R promoter by interacting with the RNA polymerase β subunit and thus regulating the initiation of λ (plasmid) replication (Szalewska-Palasz *et al.*, 1998).

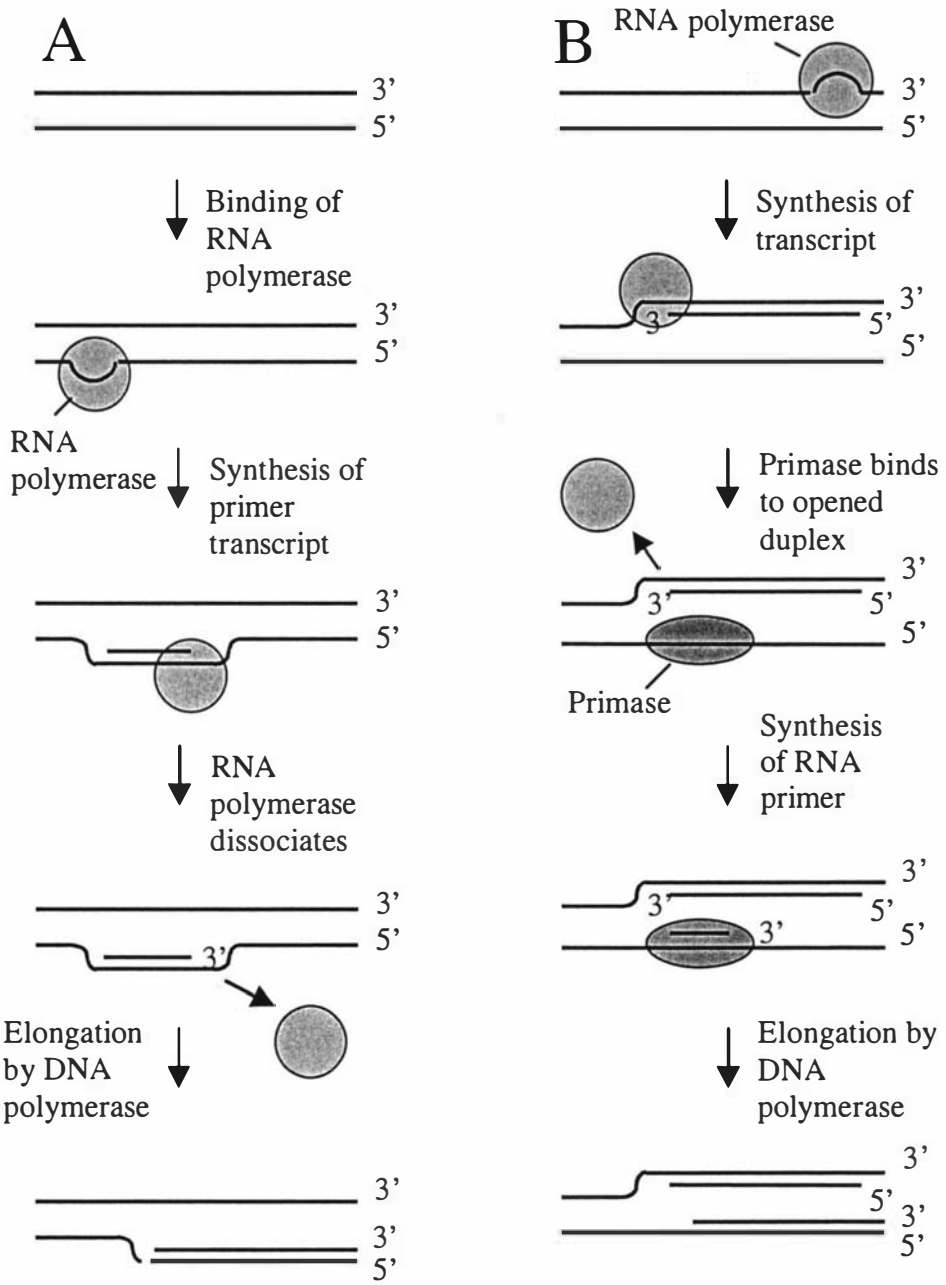


Figure 1.6. Schemes for initiation of replication by RNA polymerase.

A. RNA polymerase synthesizes the RNA primer required for replication. B. Transcription opens the double helix to allow binding of a primosome. Figure reproduced from Dasgupta *et al.* (1987).

In phage T4, several origins that are active under certain conditions have been characterized (for review see Mosig *et al.,* 1995). When cloned into plasmids, *ori(usvY)* allows for their autonomous replication. Deletion analysis has shown that *ori(usvY)* contains a middle-mode promoter followed by a DUE (DNA unwinding element; Menkens and Kreuzer, 1988). Initiation from *ori(usvY)* is rifampicin sensitive which suggests that transcription is involved in replication. The transcript made from P_M (middle-mode promoter) in *ori(usvY)* forms a persistent RNA-DNA hybrid (Carles-Kinch and Kreuzer, 1997), is required for replication, and most likely acts as a primer for replication (Belanger and Kreuzer, 1998).

RNA polymerase is known to be involved in replication of some theta replicating plasmids. An example of this is plasmid pAMβ1 replication in Gram-positive bacteria. In pAMβ1 DNA synthesis is initiated by DNA polymerase I and completed by DNA Pol III (Bruand *et al.,* 1993) and requires a plasmid-encoded protein (RepE) and a short origin (~ 44 bp). Furthermore, transcription from the *repE* promoter through the origin is also required for replication as inserting a transcriptional terminator between *repE* and the origin results in at least a ten fold reduction in the ability of the plasmid to replicate (Bruand and Ehrlich, 1998). The majority of the transcripts synthesized from *repE* terminate at the origin and are cleaved by either a host-encoded protein or RepE (which is thought to possess RNase activity). The processed transcript then serves as a primer for replication (Bruand and Ehrlich, 1998). Although it is known that RepE is required for replication, the role of the RepE protein in replication is not fully understood.

ColEI is another theta replicating plasmid that replicates in *E. coli*, and also depends on transcription by RNA polymerase. The transcript synthesized 555 bp upstream of the origin, RNAII, folds into a specific conformation to form a persistent hybrid with the template DNA (Dasgupta *et al.,* 1987; Masukata and Tomizawa, 1990). In the presence of Pol I, RNAII is cleaved by RNase H and the transcript then serves as a primer for replication (Itoh and Tomizawa, 1980). In the absence of Pol I and RNase H, lagging strand synthesis is initiated by displacing the nontranscribed strand (Masukata *et al.*, 1987). In the absence of RNase H and the presence of Pol I, the hybridized RNAII transcript can be used as a primer for leading strand synthesis without cleavage (Dasgupta *et al.,* 1987). The copy number of ColEI is regulated by a

short transcript, RNAI, which hybridizes to RNAII and thereby prevents the maturation of RNAII into the primer needed for initiation of ColEI replication (Binnie *et al.*, 1999). Alternatively, RNAI is cleaved by RNase E near its 5' end, and Poly(A)polymerase then facilitates rapid degradation of RNAI from the 3' end (Xu *et al.*, 1993).

Transcriptional activation and primer synthesis for DNA replication by RNA polymerase can also be observed in mitochondria of eukaryotes (for review see DePamphilis, 1988; mitochondria are believed to be derived from prokaryotes through the accepted theory of endosymbiosis). In mitochondria of mice, a transcript synthesized from a promoter for transcription of the light strand (LSP; Chang and Clayton, 1985) is cleaved by EndoG, and the processed transcript then acts as a primer for mtDNA replication (Côté and Ruiz-Carillo, 1993). Another RNA processing enzyme, the RNase MRP, also appears to be involved in primer maturation (Lee and Clayton, 1998).

1.4.3.1 RNA secondary and tertiary structures

Because RNAs usually occur as single-stranded molecules, base pairing can occur within an RNA molecule, which enables it to form complex secondary and tertiary structures. Many RNA species have to fold into these complex structures in order to carry out their biological functions.

Hydrogen bonds form between C-G and A-U pairs, and also between G-U pairs, although the latter are less stable. Internal loops and bulges can interrupt helical stems, and terminal (or hairpin) loops or junctions between stems can form (Figure 1.7).

RNA molecules fold into tertiary structures because unpaired bases can interact with distant stem-loop structures. Examples of this include pseudoknots, which are formed when one strand of a stem folds back to pair with bases in a loop (Figure 1.8).

RNA molecules that fold into complex structures are involved in many processes. Some examples of these processes are: transcription termination (Henkin, 1996), protein binding (Bourdeau *et al.*, 1999), translation (tRNA; ribosomal frameshifting by pseudoknots; Giedroc *et al.*, 2000), storage of genetic information of some organisms (RNA viruses and viroids), maturation of tRNA (Ribonuclease P), splicing (U RNA), regulation of plasmid copy number (ColEI), replication (primer

formation, ColEI), and cleavage of RNA (hammerhead and hairpin ribozymes; Higgs, 2000).

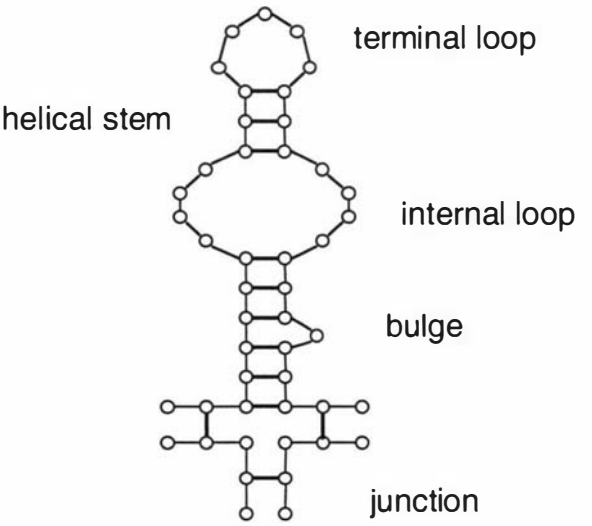


Figure 1.7. Possible RNA secondary structures.

Figure reproduced and modified from Mattaj (1993). Hydrogen bonds between bases are represented by a thick line, and thin lines represent the sugar-phosphate backbone by which the bases are joined.

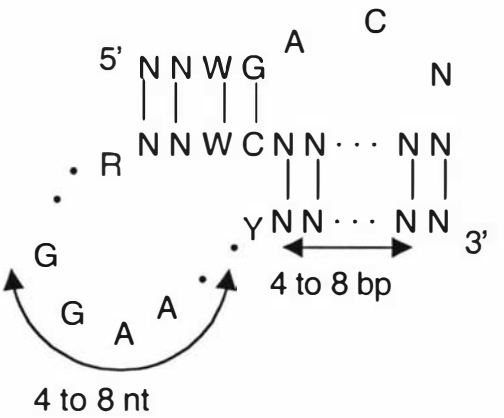


Figure 1.8. The S1-protein binding motif is a pseudoknot.

Figure reproduced from Bourdeau *et al.* (1999). N = A, C, G or U; W = A or U; R = A or G; Y = C or U.

Another important aspect of RNA folding is the relationship between RNA structure and RNA degradation. Folding of RNA into secondary structure can prevent it from being degraded by cellular ribonucleases, for example stem-loop structures at the 3' end of RNA prevent degradation by exonucleases like RNase II and PNPase (polynucleotide phosphorylase). However, endonucleases like RNase E, RNase III and RNase P can cleave the RNA internally and thus provide access for the exonucleases to RNA. Finally, multiprotein complexes (RNA degradosome) can unwind and degrade folded RNA. The RNA degradosome is composed of a PNPase, an RNase E, a DEAD-box RNA helicase and the glycolytic enzyme enolase (for review see Carpousis, 2002; Régnier and Arraiano, 2000).

The probability of an RNA molecule folding into a particular structure can be predicted by rules that describe the interactions of the base pairs. The rules are based on calculating the free energy for formation of each structure. The free energy measures the amount of energy (ΔG) absorbed or released by a reaction and is measured in Kcal/mol. Energy must be released overall to form a base paired structure; the stability of the structure is determined by the amount of free energy released.

There are many computer programs designed to predict RNA secondary structure, but there are only a few that can predict tertiary structure. However, the modelled data do not always agree with the biological data. RNA structures can be determined by nuclear magnetic resonance (NMR) and x-ray crystallography and the accuracy of the modelled structure can also be tested by using nucleases which cleave single-stranded or double-stranded RNA specifically.

1.5 Aim of the study

Extensive studies on model organisms like *E. coli* and phage λ have illuminated basic features and provided detailed information on the process of replication in these organisms. Interaction of replication proteins with other proteins and/or DNA has also been extensively studied.

At the beginning of this study little was known about lactococcal phage biology. Although substantial research had been performed at the nucleotide level (sequencing of the phage DNA and identification of genes), there was essentially no information available on lactococcal phage DNA replication. A greater understanding of the process of phage replication may lead to new possibilities for preventing or aborting phage infection of dairy lactococcal starter strains. Furthermore, a better understanding of phage DNA replication would help to understand the co-evolution of phage and their hosts.

Previous work identified the c2 origin of replication (Lubbers *et al.*, 1995; Waterfield *et al.*, 1996) and identified several untranslated transcripts in the origin region (Lubbers *et al.*, 1998). This suggests that transcription is involved in phage c2 replication. Although it is not uncommon for transcription to be implicated in replication, the role of transcription in phage c2 DNA replication was not clear at the outset of the study. Therefore the aims of this project were:

(i) identification of the minimal c2 *ori,*
(ii) investigation of the requirement for a functional promoter in the c2 origin, and therefore determination of the importance of transcription in replication,
(iii) characterization of the transcripts synthesized from the early promoter 1 (P_E1) in the c2 *ori,* investigation of their involvement in replication, and their sequence requirements in order to support replication, and
(iv) identification of lactococcal host factors involved in c2 replication.

For ease of manipulation, the above objectives were addressed using a plasmid model system, whereby the c2 *ori* and derivatives thereof were tested for their ability to support plasmid replication.

2. MATERIALS AND METHODS

2.1 Bacterial strains, phage, culture and storage conditions

Bacterial strains and phage used in this study are listed in Table 2.1.

Table 2.1. Bacterial strains and phage.

Bacterial strain, phage	Characteristics or genotype	Source/reference
E. coli ER2206	*endA1 thi1 supE44 mcr67 (mrcA⁻) (mrcBC-hsdRMS-mrr)114*::IS*10 (lac)U169/F' proAB laqI*^{q} *ZDM15* Tn*10* (Tet^{r})	New England Biolabs, Beverly, USA
L. lactis subsp. *cremoris* MG1363	plasmid-free strain and prophage cured derivative of NCDO 712	Gasson, 1983
L. lactis subsp. *cremoris* NZ9000	Strain MG1363 with *pepN:nisR nisK*, non-nisin producer	Kuipers *et al.*, 1998
L. lactis subsp. *cremoris* NZ9800	NZ9700 derivative; *nisBTCIPRKFEG*, non-nisin producer	Kuipers *et al.*, 1993
c2	prolate-headed, c2 species	Pillidge and Jarvis, 1988

Escherichia coli was cultured on Luria-Bertani agar (LBA) and brain heart infusion agar (BHI-A), and in Luria-Bertani broth (LB) and brain heart infusion broth (BHI-B) with aeration (shaker at 200 rpm) at 37°C (see Table 2.2). Stocks were maintained at -70°C in LB containing 35% glycerol.

Lactococcus lactis cultures were grown at 30°C without aeration in M17 medium supplemented with 0.5% (w/v) glucose (GM17, Table 2.2). Stock cultures were stored at -70°C in GM17 containing 30% glycerol.

2.2 Media and supplements

All media were prepared using water which had been purified by the MilliQ Reagent Water System (Millipore). The media were then sterilized at 121°C for 20 min. Media prepared for bacterial culture during this study are listed in Table 2.2.

Table 2.2. Media.

Medium	Recipe	Reference/supplier
Luria-Bertani broth (LB)	1% (w/v) tryptone (Invitrogen, Carlsbad, USA), 0.5% (w/v) yeast extract (Merck, Darmstadt, Germany), 0.5% (w/v) NaCl; adjust pH to 7.0 with NaOH	(Sambrook *et al.*, 1989)
Luria-Bertani agar (LBA)	1% (w/v) tryptone (Invitrogen, Carlsbad, USA), 0.5% (w/v) yeast extract (Merck, Darmstadt, Germany), 0.5% (w/v) NaCl; adjust pH to 7.0 with NaOH, 1.5% (w/v) bacto-agar (Oxoid, Basingstoke, England)	(Sambrook *et al.*, 1989)
Brain heart infusion broth (BHI-B)	3.7% (w/v) brain heart infusion (Difco, Detroit, MI, USA)	Difco, Detroit, USA
Brain heart infusion agar (BHI-A)	3.7% (w/v) brain heart infusion (Difco, Detroit, MI, USA), 1.5% (w/v) bacto-agar (Oxoid)	Difco, Detroit, USA
M17	3.7% (w/v) M17 (Difco)	Difco (Terzaghi and Sandine, 1975)
GM17	3.7% (w/v) M17 (Difco), 0.5% (w/v) glucose	Difco
GM17 agar	3.7% (w/v) M17 (Difco), 0.5% (w/v) glucose, 1.5% (w/v) bacto-agar (Oxoid)	Difco
GM17 soft agar	3.7% (w/v) M17 (Difco), 0.5% (w/v) glucose, 0.45% (w/v) bacto-agar (Oxoid)	Difco

Stock solutions of antibiotics and supplements were kept at -20°C in sterile MilliQ-treated water, unless otherwise stated. For preparation of solid media, the supplements and antibiotics were mixed with liquified media, which had been cooled to 55°C. The supplemented media were poured immediately after mixing. All growth media supplements and antibiotics are listed in Table 2.3.

Table 2.3. Media supplements and antibiotics.

Supplement	Stock concentration [mg/ml]	Final concentration [µg/ml] for:	
		L. lactis	*E. coli*
Ampicillin (Roche, Mannheim, Germany)	100	n/a[a]	100
Chloramphenicol (Roche, Mannheim, Germany)	20[b]	5	25
Erythromycin (Sigma, St. Louis, USA)	50[c]	5	100[d]
IPTG[e] (AppliChem, Darmstadt, Germany)	240	n/a	48
X-gal[f] (AppliChem, Darmstadt, Germany)	20[g]	n/a	32
Nisaplin (Aplin & Berrett, Beaminster, United Kingdom)	10[h]	0.005 - 0.150	n/a

[a]not applicable

[b]dissolved in ethanol

[c]dissolved in methanol

[d]BHI-A and BHI-B were used when selecting for erythromycin resistance

[e]Isopropyl-β-D-thiogalactopyranoside

[f]5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

[g]dissolved in dimethylformamide, wrapped in foil for storage

[h]dissolved in 0.1 M HCl, 10^6 units nisin g^{-1}, stored at 4°C

2.3 Phage c2 propagation

2.3.1 Preparation of phage lysate

To prepare phage lysate, 10 ml of GM17 (see section 2.2) was inoculated with 100 µl of a *L. lactis* MG1363 overnight (ON) culture and the cells grown at 30°C to an OD_{600} of 0.1. $CaCl_2$ to a final concentration of 5 mM was then added followed by 1 ml of phage c2 lysate (10^7-10^8 pfu/ml). The mixture was then incubated at 30°C until the culture cleared (approximately one hour). Any remaining cells were removed by centrifuging the lysate at 10,000 rpm for 10 min in a SS34 rotor (RC-5B, Sorvall centrifuge). The resultant supernatant was filtered through a sterile 0.45 µm filter (Millipore) and stored at 4°C.

2.3.2 Titration of phage lysate

GM17 soft agar (Table 2.2) was melted, and 2.5 ml aliquots were transferred into glass test tubes and kept at 50°C. $CaCl_2$ was added to the tubes to a final concentration of 5 mM, the tubes vortexed, and 200-300 µl of *L. lactis* MG1363 ON culture added. The media and bacteria were then mixed gently, and poured onto GM17 agar plates. While the plates were drying, several dilutions of the phage were prepared (i.e. $1x10^{-2}$, $1x10^{-4}$, $1x10^{-6}$) in diluent (10% M17). 10 µl aliquots of the phage dilutions were spotted onto the plates and dried before incubating ON at 30°C. The plaques were counted and the plaque forming units per ml [pfu/ml] were determined.

2.4 Oligonucleotide primers

Oligonucleotides used in this study and their priming orientations (relative to the plasmid sequences), positions and applications are listed in Table 2.4. Primers were obtained from Life Technologies (Carlsbad, USA) or Sigma Genosys in deprotected form. The oligonucleotides were supplied without extra purification, unless otherwise stated.

Table 2.4. Oligonucleotide primers.

Primer	Sequence 5'→3'	Priming position	Application
pUC/M13 RP	CAG GAA ACA GCT ATG AC	pUC18/19; ←	sequencing primer for *ori*-fragments cloned in pUC19; orientation of inserts cloned in pFX3
pUC/M13 FP	GTT TTC CCA GTC ACG AC	pUC18/19; →	sequencing primer for *ori*-fragments cloned in pUC19; orientation of inserts cloned in pFX3
LX-1	GAA CAC TAT CCC ATA CAC C	Pos. 2677-2696 in pVA891; →	sequencing primer for *ori*-fragments cloned in pVA891; orientation of inserts
LX-2	GCC TTT ATT CAC ATT CTT GC	Pos. 2751-2770 in pVA891; ←	sequencing primer for *ori*-fragments cloned in pVA891; orientation of inserts
LP206	TTT GAA TTC AAA CGC AGT TTT TAT CCC	Pos. 6749-6768 in c2; 3' end of P_E1 transcript; + *Eco*RI; →	deletion analysis of c2 *ori*
LP207	GGG GAA TTC TCA CAT CAA TTC GGC	Pos. 6817-6831 in c2; 3' end of P_E1 transcript; + *Eco*RI; →	deletion analysis of c2 *ori*
LP208	GGG GAA TTC AAC TTT GCC TAT GTT GGG	Pos. 6865-6882 in c2; 3' end of P_E1 transcript; + *Eco*RI; →	deletion analysis of c2 *ori*
LP209	TTT GAA TTC CTT TGC CTA ACT CAT TAC	Pos. 6928-6945 in c2; 3' end of P_E1 transcript; + *Eco*RI; →	deletion analysis of c2 *ori*
LP210	GGG GAA TTC GTA CGG TTT TCA TAT ACT C	Pos. 6960-6978 in c2; 3' end of P_E1 transcript; + *Eco*RI; →	deletion analysis of c2 *ori*
LP211	TTT GAA TTC ACA AGC CTT AGG CGT ATT C	Pos. 6996-7014 in c2; 3' end of P_E1 transcript; + *Eco*RI; →	deletion analysis of c2 *ori*
latedel	AAA GAA TTC CTT GTA TTT TTG ACC CTG	Pos. 7068-7085 in c2; upstream of P_E1; + *Eco*RI; ←	deletion analysis of c2 *ori;* P_E1 modifications of c2 *ori*
6902prex	TTC AGT GAC ATC ACA CAG GGC	Pos. 6902-6922 in c2; downstream of P_E1; →	primer extension (PAGE purified)
AS1	ACG TAT GTT ATA ATG TAA AAA AAT CAG GGT CA	Pos. 7058-7073 in c2; P_E1; →	P_E1 modifications of c2 *ori*
AS2	TTT TTC AAA ATC AAA AAT ATA TAA AAA TTG AAT AC	Pos. 7009-7029 in c2; P_E1; ←	P_E1 modifications of c2 *ori*
AS5	AAA GAA TTC ACT ATC GAC AAT CTT ACC GTC	Pos. 7878-7896 in pHP003; + *Eco*RI; ←	PCR amplification of *prtP* A-domain
AS6prot	GAT CAA ATG GAC AGT AAT ACG G	Pos. 7601-7621 in pHP003; →	PCR amplification of *prtP* A-domain

Table 2.4 continued

AS7prot	AAG TAT TAC TGT CCA TTT GAT CAA TTT TTA TAT ATT TAT TAT AAC	Pos. 7014-7038 in c2; ←	P_E1 transcript analysis
207Atermin	CAT GCC ATG GCT TGT ATT TTT GAC CCT GA	Pos. 7067-7085 in c2; upstream of P_E1; + *Nco*I; →	P_E1 transcript analysis
PstI207termin	AAA ACT GCA GTC ACA TCA ATT ACG G	Pos. 6817-6830 in c2; 3' end of P_E1 transcript; + *Pst*I; →	PCR amplification of pLP207
NcoI207termin	TTC ATG CCA TGG CTT GTA TTT TTG ACC	Pos. 7071-7086 in c2; upstream of P_E1; + *Nco*I; ←	PCR amplification of pLP207
JR225	TCG GAA TTC CAC GCA AAC GCA GTT TTT ATT CTT GAA CTA ATT AAT ATG TC	Pos. 6746-6793 in c2; 3' end of P_E1 transcript; + *Eco*RI; →	P_E1 transcript analysis
JR190	CGT ATT CAA TTT TTA TTC CTA TAG TGA GTC GTA TTA C	Pos. 3176-3197 in pGEM®-3Zf; T7 promoter; →	transcript analysis, sense probe
JR194	CGT TAC AAT TTC CAT TCG CC	Pos. 3007-3026 in pGEM®-3Zf; T7 promoter; ←	transcript analysis, sense probe
JR198	CTA TAG GAA TAA AAA TTG AAT ACG CCT AA	Pos. 7003-7023 in c2; +1 of P_E1 transcript; ←	transcript analysis, sense probe
JR199	AAA CGC AGT TTT TAT CCC C	Pos. 6751-6768 in c2; 3' end of P_E1 transcript; →	transcript analysis, sense probe
JR200	TCA CAT CAA TTC GGC TAT G	Pos. 6817-6835 in c2; 3' end of P_E1 transcript; →	transcript analysis, sense probe
LPS	TAG TTA CCT TGC TAA AGG G	Pos. 6717-6735 in c2; 3' end of P_E1 transcript; →	transcript analysis, sense probe
LPA	CGA ATT CAT TGA TTA TGG TAT TAT T	Pos. 7213-7230 in c2; P_L1; ←	transcript analysis

2.5 Vectors and recombinant plasmids

Vectors and recombinant plasmids constructed or used in this work are listed in Table 2.5.

Table 2.5. Plasmids used in this study.

Plasmid	Characteristics	Reference/source
pFX3	cm, high-copy number shuttle vector between *Escherichia coli* and *Lactococcus*, MCS	Xu *et al.*, 1991a
pTRKL2	ery, low-copy number shuttle vector between *Escherichia coli*, *Lactococcus*, *Enterococcus*, *Streptococcus* and *Lactobacillus*	O'Sullivan and Klaenhammer, 1993
pVA891	ery,cm, tet, low-copy number shuttle vector between *Escherichia coli* and *Streptococcus*	Macrina *et al.*, 1983

Table 2.5 continued

pUC19	amp, ColE1 origin, blue/white, MCS	Yanish-Perron *et al.*, 1985
pGEM®-3Zf⁻	amp, blue/white, MCS	Promega, Madison, USA
pNZ8037	cm, MCS	De Ruyter *et al.*, 1996b
pLP201	ery, tet, 521 bp *ori*-fragment from c2 cloned into pVA891	Waterfield *et al.*, 1996
pLP203	ery, tet, based on pLP201, P_L1 deleted	Gordon (1997)
pLP204	ery, tet, based on pLP201, P_E1 deleted	Gordon (1997)
pLP205	ery, tet, based on pLP201, T→G, T→G base pair mutations in -10 region of P_E1	M. Callanan
pLP206	ery, tet, based on pLP203, 3' end deletion of transcript made from P_E1	this study
pLP207	ery, tet, based on pLP203, 3' end deletion of transcript made from P_E1	this study
pLP208	ery, tet, based on pLP203, 3' end deletion of transcript made from P_E1	this study
pLP209	ery, tet, based on pLP203, 3' end deletion of transcript made from P_E1	this study
pLP210	ery, tet, based on pLP203, 3' end deletion of transcript made from P_E1	this study
pLP211	ery, tet, based on pLP203, 3' end deletion of transcript made from P_E1	this study
pLP212	ery, tet, based on pLP201, P_E1 replaced by *nisA* promoter	M. Callanan
pLP213	ery, tet, based on pLP203, inversion of P_E1	this study
pLP214	ery, tet, based on pLP203, transcript synthesized from P_E1 replaced by unrelated lactococcal sequence	this study
pLP215	ery, tet, base pair deletions (7 G residues) in the P_E1 transcript	this study
pLP216	ery, tet, c2 *ori* fragment of pLP207 cloned into pVA891Ω	this study
pUC4Ωkm-2	amp, kan, *Streptococcus faecalis aphA-3* gene in Ω element (including terminator) cloned in pUC4	Perez-Casal *et al.*, 1991
pUC19-*ori*	amp, 521 bp *ori* fragment from c2 cloned into pUC19	Waterfield *et al.*, 1996
pUC19-203	amp, c2 *ori* insert of pLP203 cloned into pUC19	Gordon (1997)
pFX3-203	cm, c2 *ori* insert of pLP203 cloned into pFX3	J. Rakonjac
pFX3-204	cm, c2 *ori* insert of pLP204 cloned into pFX3	this study
pFX3-206	cm, c2 *ori* insert of pLP206 cloned into pFX3	this study
pFX3-207	cm, c2 *ori* insert of pLP207 cloned into pFX3	this study
pFX3-208	cm, c2 *ori* insert of pLP208 cloned into pFX3	this study
pFX3-213	cm, c2 *ori* insert of pLP213 cloned into pFX3	this study
pFX3-214	cm, c2 *ori* insert of pLP214 cloned into pFX3	this study
pFX3-215	cm, c2 *ori* insert of pLP215 cloned into pFX3	this study
pFX3-216	cm, c2 *ori* insert of pLP216 cloned into pFX3	this study
pT7-207	amp, pUC19, T7 promoter, 207 nt P_E1 transcript, sense probe	this study
pT7-307	amp, pUC19, T7 promoter, 307 nt P_E1 transcript, sense probe	this study
pHP003	lactocepin plasmid from *L. lactis* HP containing the *prtP* gene	Christensson *et al.*, 2001
pVA891Ω	ery, tet, pVA891 + Ω terminator from pUC4Ωkm-2	Rakonjac *et al.* 2002 (submitted)
pGEMc2	amp, c2 antisense probe cloned into pGEM®-3Zf⁻	Rakonjac *et al.*, 2002 (submitted)

2.6 DNA isolation

2.6.1 Plasmid preparation

2.6.1.1 Alkaline lysis plasmid preparation

Alkaline lysis preparation of *E. coli* plasmids was performed essentially as described by Sambrook *et al.* (1989). A brief summary of the procedure is described below. 1.5 ml of ON *E. coli* culture was pelleted by centrifugation at 14,000 rpm (20,800 xg) at room temperature (RT) for 30 sec using a bench-top centrifuge (5417C, Eppendorf). The supernatant was removed by aspiration and the pellet resuspended in 100 µl of resuspension buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0). 200 µl of lysis buffer (0.2 N NaOH, 1% (w/v) SDS) was then added and the tubes mixed gently by inversion. This was followed by the addition of 150 µl of neutralization buffer (3 M potassium, 5 M acetate). The tubes were then remixed gently by inversion and cooled on ice for 3-5 min. After cooling, the samples were recentrifuged for 10 min at 14,000 rpm (20,800 xg) and the supernatant transferred to a fresh tube. The plasmid DNA was precipitated with 0.7 volumes of isopropanol (RT for 5 min) and the samples recentrifuged for 10 min at 14,000 rpm (20,800 xg). The supernatant was removed by aspiration and the pellet washed with 100 µl of 70% ethanol and air dried. In the final step, the plasmid DNA was dissolved in 40-100 µl sterile MilliQ water or 1x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

2.6.1.2 High Pure™ plasmid preparation

For preparation of high purity plasmid DNA, the High Pure™ Plasmid Isolation Kit (Roche, Mannheim, Germany) was used according to the manufacturer's instructions. In this method, bacterial cells were lysed, and the plasmid DNA bound selectively to glass fiber fleece in the presence of a chaotropic salt. After sequential washes in washing buffer 1 and 2 (Roche, Mannheim, Germany), the DNA was eluted with either sterile MilliQ water or elution buffer (10 mM Tris-HCl, pH 8.5). Using this

method, a 1.7 ml ON *E. coli* culture routinely yielded 10 μg of pure high copy plasmid DNA.

2.6.1.3 Preparation of plasmid DNA from *L. lactis*

Plasmid DNA from *L. lactis* was isolated according to the method of Anderson and McKay (1983); a summary of the modified procedure is described below. 40 ml of *L. lactis* culture was grown at 30°C to an OD_{600} of 0.7 (Nova Tech spectrophotometer). The bacteria were then pelleted by centrifugation at 5,000 rpm at 4°C in a SS34 rotor (RC-5B Sorvall centrifuge) and the cells resuspended in 1.8 ml of resuspension buffer (6.7% (w/v) sucrose, 50 mM Tris-HCl, 1 mM EDTA, pH 8.0). The suspension was then warmed to 37°C, and 480 μl of lysozyme (10 mg/ml in 25 mM Tris-HCl, pH 8.0) added. After incubation of the suspension at 37°C for 10 min, 240 μl of buffer 2 (0.25 M EDTA, 50 mM Tris-HCl, pH 8.0) and 138 μl of buffer 3 (20% (w/v) SDS in 50 mM Tris-HCl, 20 mM EDTA, pH 8.0) was added. The cells were mixed and incubated for 10 min at 37°C to complete lysis, 138 μl of buffer 4 (freshly prepared 3.0 N NaOH) added, and the samples mixed gently for 5 min. 248 μl of buffer 5 (2.0 M Tris-HCl, pH 7.0) was then added and the samples mixed for a further 3 min. This was followed by the sequential addition of 358 μl of buffer 6 (5.0 M NaCl) and 3.5 ml of phenol saturated with 3% NaCl. After centrifugation at 5,000 rpm at RT in a SS34 rotor (RC-5B Sorvall centrifuge), the upper phase was transferred to a fresh tube and the DNA extracted with 3.5 ml of chloroform-isoamyl alcohol. After recentrifuging the samples at 5,000 rpm at RT in a SS34 rotor, the upper phase (DNA) was then transferred to a new tube and the plasmid DNA precipitated with 1 vol of isopropanol. The samples were kept at -20°C for 30 min and centrifuged at 8,000 rpm at 4°C for 20 min in a SS34 rotor. The pellet was then washed in 70% ethanol and the plasmid DNA dissolved in 1x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

2.7 DNA analysis methods

2.7.1 DNA agarose gel electrophoresis

DNA was separated on horizontal agarose gels (Horizon® 58 mini gel apparatus; Invitrogen, Carlsbad, USA) and visualized according to Sambrook *et al.* (1989). Agarose in the gels was at 0.8% (w/v), 1% (w/v) and 2% (w/v) in 1x TAE buffer (40 mM Tris, 19 mM acetic acid, 1 mM EDTA). Before loading, the DNA samples were mixed with 0.2 vol DNA loading dye (0.5% (w/v) bromophenol blue, 0.5% (w/v) xylene cyanol, 50 mM EDTA, 15% (w/v) Ficoll (type 400)). Electrophoresis was performed at 60-100 V for 40-90 min. The gel was stained in 5 μg/ml ethidium bromide solution for 10-20 min and then destained in water for 5 min. The DNA was then visualized under UV irradiation using the TMW-20 Transilluminator (Alpha Innotech), and images captured by the Alpha Imager 2000 System (Alpha Innotech).

The molecular mass of DNA fragments was determined by direct comparison of their migration distance to that of a set of standard fragments of known size run on the same agarose gel. The 1 kb Plus and the supercoiled ladder (both Invitrogen, Carlsbad, USA) were used as standard molecular markers according to the manufacturer's instructions.

2.7.2 DNA restriction endonuclease treatment

The restriction endonucleases used in this study were supplied by Roche (Mannheim, Germany) and Invitrogen (Carlsbad, USA). DNA was digested using the appropriate buffer for 2 hours or ON according to the manufacturer's instructions. To ensure low glycerol concentrations, the volume of the added enzyme never exceeded 10% of the total reaction volume. When two enzymes were used simultaneously in a digest, a buffer ensuring sufficient activity for both enzymes was used. If the optimal temperatures for the two enzymes were different, an hour incubation at the lower of the two temperatures was used followed by an hour incubation at the higher optimal

temperature. The restriction digest was examined by gel electrophoresis (section 2.7.1) of a small aliquot of the digestion mix.

2.7.3 DNA quantification

DNA concentrations were quantified by spectrophotometric analysis at 260 nm and 280 nm using an Ultrospec® 3000 (Amersham Pharmacia Biotech, Little Chalfont, England) spectrophotometer and quartz cuvettes (Starna) with a 1 cm light path. DNA concentrations [μg/ml] were calculated by multiplying any dilution factors, with a constant factor (50 for dsDNA), and the absorbance reading at 260 nm. A measure of the purity of the samples was calculated by the ratio of the absorbances at 260 nm and 280 nm. Samples with ratios ≥1.8 were considered to be of high purity and low protein contamination.

When the DNA concentration was low (<100 ng/μl), the concentration was determined using densitometry in agarose gels by comparison to six standard solutions of phage λ DNA (Invitrogen, Carlsbad, USA) of various concentrations (15 ng-500 ng). Alternatively the 1,650 bp band in the 1 kb Plus DNA ladder (Invitrogen, Carlsbad, USA) was used for calculating the concentration of DNA. The 1,650 bp band contains 8% of the ladder mass applied to the gel.

2.7.4 DNA sequencing

DNA was sequenced by MUseq DNA Analysis Service (Massey University, Palmerston North, New Zealand) on an ABI Prism™ 377 DNA sequencer. Templates were prepared using the High Pure™ Plasmid Isolation Kit (Roche,Mannheim, Germany section 2.6.1.2). Typically 4 μl of 0.8 pmol/μl primer and 5 μl of 200-300 ng/μl plasmid DNA were used per sequencing reaction. Sequencing reactions were performed using dye terminator chemistry based on the technique described by Sanger *et al.* (1974). Generated data were visualized as chromatograms using ABI Prism software (Editview).

Sequence data were also generated using the AmpliCycle™ Sequencing Kit (Perkin Elmer) following the instructions of the manufacturer. 20 pmol of primer was end-labelled with [γ-^{32}P] ATP (Amersham Pharmacia Biotech, Little Chalfont, England) using T4 polynucleotide kinase according to the instructions of the Primer Extension System-AMV Reverse Transcriptase kit (Promega, Madison, USA). 4 pmol of the end-labelled primer was used per sequencing reaction.

Sequencing reactions were separated on a denaturing polyacrylamide gel using a model S2 sequencing apparatus (Invitrogen, Carlsbad, USA). The polyacrylamide gel was prepared as follows: an acrylamide stock solution consisting of 38% acrylamide and 2% N, N' methylenebisacrylamide was prepared and then deionized by adding 50 g/l Amberlite MB-3 matrix. The solution was stirred for 30 min at RT, then filtered through 3 MM chromatography paper (Whatman) and stored in the dark at 4°C. 33 g urea, 7.7 ml 10x TBE (0.89 M Tris, 0.89 M boric acid, 20 mM EDTA), 13 ml acrylamide stock solution, 33 ml water, 370 µl of 10% (w/v) ammonium persulfate, and 37 µl TEMED (crosslinking agent) were mixed together. The mix was poured into the gel holder and stored until polymerization was complete. Using 1x TBE as the running buffer, the gel was pre-run for 45 min at 1,500 V and then the samples run for 2-3 hours at 1,500 V. The gel was fixed in 10% acetic acid for 30 min, dried for 1 hour at 80°C in a gel dryer (model 583, Bio-Rad) and exposed to x-ray film (Fuji Super RX). The x-ray film was developed after exposure for up to 5 days in an automatic x-ray film processor (100 Plus™, All Pro Imaging).

The sequencing data were analyzed using Geneworks (IntelliGenetics, Mountain View, CA, USA) and the GeneJockey analysis program (Biosoft, Ferguson, MO, USA), and database searches were performed on the server of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm (Altschul *et al.*, 1990).

2.8 Polymerase chain reaction (PCR)

PCR was performed with custom primers according to the recommendations of the DNA polymerase supplier. DNA was amplified using two oligonucleotides flanking the region of interest. The template DNA was first denatured using high temperature, the primer annealed specifically to its target sequence (temperature dependent on the primers) and then the DNA was synthesized (72°C). The cycle was repeated 25-30 times.

Pwo polymerase creates blunt ended DNA with higher fidelity than *Taq* polymerase i.e. it has a lower misincorporation rate. Thus 0.5 U of *Pwo* polymerase (Roche, Mannheim, Germany) was used per reaction for amplifying the DNA fragments which were used for cloning and all other reactions were performed using 0.5 U of *Taq* polymerase (Roche, Mannheim, Germany). Usually, total reaction volumes of 10 µl or 20 µl were used. The *Taq* polymerase reaction mixture contained a final concentration of 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.4 µM primer and 1x reaction buffer. The *Pwo* polymerase reaction mixture was identical to the *Taq* polymerase reaction mixture except the $MgCl_2$ was substituted by 2.0 mM $MgSO_4$. Typically around 5-10 ng of template DNA was used per reaction. PCRs were performed in 200 µl thin wall reaction tubes in an FTS-960 Microplate Thermal Sequencer (Corbett Research).

A typical reaction consisted of an initial denaturation step for 2 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec primer annealing at 53°C (variable, see below), 45 sec (variable, see below) extension at 72°C, and a final incubation for 5 min at 72°C. The reaction mixtures were stored at 4°C. The annealing temperatures of the primers were always approximately 2°C lower than their calculated melting temperatures (T_m). The melting temperature was calculated as follows: $T_m = 69.3 + 0.41 \times (GC\%) - 650/n$, where n is the number of nucleotides of the primer and GC% is the percentage of G- and C-nucleotides in the primer sequence. Extension times were calculated according to the expected size of the product assuming a polymerization rate of 30-40 bp/sec (Sambrook *et al.*, 1989).

2.9 Cloning techniques

Essentially, cloning consisted of four steps: preparation of the DNA fragment to be cloned and the vector DNA, ligation, transformation of the host cell with the ligation product, and finally screening for desired recombinants using the alkaline lysis plasmid preparation (section 2.6.1.1).

2.9.1 Vector and insert preparation

Vector DNA used in cloning was purified using the High Pure™ Plasmid Isolation Kit (Roche, Mannheim, Germany), treated with a restriction endonuclease (section 2.7.2.), dephosphorylated (calf intestinal alkaline phosphatase, CIP, New England Biolabs, Beverly, USA), and then separated by agarose gel electrophoresis. After electrophoresis, the DNA was stained in ethidium bromide, visualized using the Mineralight ® lamp (Model UVGL-58) and then excised from the gel. The DNA was purified using the High Pure™ PCR Product Purification Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. A brief outline of the method is described below. The gel fragment containing the DNA was heated for at least 10 min at 56°C in binding buffer (300 µl/100 mg gel), isopropanol added (150 µl/100 mg gel), and the mixture vortexed and then applied to the column. In the presence of chaotropic salts (binding buffer), the DNA bound specifically to silica-gel particles in the column and after two washes, the DNA was eluted using either water or a low salt solution (i.e. 50-100 µl water or 50-100 µl of 1 mM Tris-HCl, pH 8.5).

The insert DNA was prepared either by PCR using *Pwo* polymerase (see section 2.8) and subsequent digestion with restriction endonucleases, or by digestion of plasmids and isolation of the DNA fragment of interest. Recovery from the agarose gel was performed as described for the vector DNA. The termini of the vector and insert DNA were designed to be cohesive to ensure efficient ligation.

2.9.2 Ligation

For the ligation, 1 U of T4 DNA ligase (Roche, Mannheim, Germany) and 1x ligation buffer (supplied with the T4 DNA ligase) were used per reaction and the total reaction volume was 10 µl. Ligation of DNA fragments with cohesive termini were incubated ON at 4°C, while ligations with blunt-ended DNA molecules were incubated ON at 16°C. Usually approximately 20 ng of insert DNA was used per ligation. A three-fold molar excess of insert DNA to vector DNA was used to ensure an adequate level of vector/insert ligation.

2.9.3 Transformation

2.9.3.1 Preparation of competent cells

For long-term storage, competent *E. coli* cells were prepared using a modified protocol of Hanahan (1983). An ON culture of *E. coli* was diluted fifty fold in LB and then grown to an OD_{600} of 0.5. The cells were pelleted at 4°C for 5 min at 7,000 rpm in a GSA rotor (RC-5B Sorvall centrifuge) and then kept on ice. The supernatant was discarded and the pellet resuspended in sterile ice cold buffer 1 (10 mM sodium acetate, 50 mM $MnCl_2$, 5 mM NaCl, pH 5.6) using 1/5 of the original culture volume and kept on ice for 20 min. The cells were harvested by centrifugation as described above and resuspended in sterile ice cold buffer 2 (10 mM sodium acetate, 5 mM $MnCl_2$, 5% glycerol, 70 mM $CaCl_2$, pH 5.6) using 1/25 of the original culture volume. The resuspended cells were aliquoted into sterile Eppendorf tubes (100 µl/tube) and stored immediately at -80°C. Typically 100 µl of competent cells were used per transformation. Transformation frequency of the cells was determined using 10 ng of pUC19 DNA and was calculated as follows:

transformation efficiency [cfu/µg] =

$$\frac{\text{colony forming units on plate}}{\text{ng of plasmid used}} \times \frac{10^3 \text{ ng}}{1 \text{ µg}} \times \text{final dilution plated}$$

The transformation frequency of the *E. coli* cells was typically 10^6-10^7 transformants/μg DNA.

Alternatively, competent *E. coli* cells were prepared according to the method of Chung *et al.* (1989). 50 ml LB was inoculated with a single colony of *E. coli* and grown to an OD_{600} of 0.4-0.5. The cells were pelleted by centrifugation at 2,500 xg for 15 min at 4°C and then gently resuspended in 5 ml of ice-cold TSS buffer (LB broth containing 10% polyethylene glycol MW 3350, 5% dimethyl sulfoxide, 50 mM $MgCl_2$, pH 6.5). After resuspension, the cells were placed on ice and used immediately (100 μl per transformation). No heat shock was required to transform the bacteria. The competence of the cells was determined as described above and the transformation efficiency of these *E. coli* cells was typically 10^7–10^8 transformants/μg DNA.

Electrocompetent cells of *L. lactis* were prepared according to a protocol based on Holo and Nes (1989). 1 ml of an ON culture of *L. lactis* was added to 90 mls of 1.25x GM17 supplemented with 10–14 ml of 25% (w/v) glycine. The cells were grown ON at 30°C to an OD_{600} of 0.5–0.6. The cells were chilled on ice for 30 min and then pelleted at 3,000 rpm for 15 min at 4°C using a GSA rotor (RC-5B Sorvall centrifuge). The supernatant was discarded and the pellet resuspended in 50 ml of ice cold resuspension buffer (0.5 M sucrose, 5 mM $KHPO_4$, 1 mM $MgCl_2$, pH 7.4). The cells were pelleted at 3,000 rpm for 10 min at 4°C using a GSA rotor, resuspended in 20 ml of resuspension buffer and centrifuged as in the previous step. The pellet was then resuspended in 10 ml of storage solution (0.5 M sucrose, 10% glycerol) and 100 μl aliquots of the competent cells transferred into Eppendorf tubes and stored at -80°C. The transformation efficiency of the *L. lactis* cells was between 10^4–10^5 transformants/μg DNA.

2.9.3.2 Transformation procedure

The $CaCl_2$ competent *E. coli* cells were transformed according to the method described by Sambrook *et al.* (1989). A short summary of the method is described below. 100 μl of competent cells were thawed on ice, the DNA added and the mixture

incubated on ice for 30 min. The cells were heat-shocked for 1 min at 42°C and reincubated on ice for 2 min. 900 µl of LB was added and the mixture shaken (200 rpm) at 37°C for 1 hour. 100-200 µl of the transformation mixture was then plated onto LB agar plates containing the appropriate antibiotics (BHI agar plates were used for the selection of erythromycin resistance) and the plates incubated ON at 37°C.

In accordance to the method described by Chung *et al.* (1989), the competent cells and DNA were incubated for 30 min on ice and then 900 µl TSS buffer (see section 2.9.3.1) added to the mixture. The cells were grown at 37°C with shaking (200 rpm) for one hour and then plated on LB or BHI agar plates containing the appropriate antibiotics. The plates were incubated ON at 37°C.

To eliminate salts present in the ligation buffer, the ligation mixtures were precipitated and resuspended in water prior to electroporation; precipitation was not necessary for plasmid DNA dissolved in water. 100 µl of electrocompetent *L. lactis* cells were thawed on ice, and the DNA chilled on ice in an Eppendorf tube for at least 5 min. DNA was added to the chilled cells (500 ng DNA per electroporation), gently mixed, and then transferred to a chilled cuvette (2 mm, ECU-102, EquiBio). For electroporation, the Gene Pulser (Bio-Rad) was operated at 200 Ω, 2.5 kV and 25 µF. The electroporated cells were transferred to an Eppendorf tube containing 950 µl recovery medium (1% glucose, 0.5 M sucrose, 1.25x GM17), incubated at 30°C for two hours and then plated onto GM17 agar plates containing the appropriate antibiotic. If erythromycin was used as the selective antibiotic, it had to be added to the recovery medium in order to induce resistance (0.005 µg/ml final erythromycin concentration); other antibiotics did not require induction. Antibiotic resistant colonies were obtained after incubation at 30°C for 16-48 hours. For every electroporation experiment, a positive control was included (pFX3 or pTRKL2). The correlation between the amount of DNA used per electroporation and the transformation frequency was linear.

2.10 RNA Isolation and analysis

2.10.1 General precautions for handling RNA

To prevent RNase contamination, the guideline provided by Sambrook *et al.* (1989) was followed. At all times disposable gloves were worn and a special work area was designated for RNA work only. All glassware was baked at 180°C for 4 hours and RNase*AWAY*™ reagent (Molecular Bio-Products, San Diego, USA) was used to clean the working area and all plasticware. All solutions (except solutions containing Tris) were made with DEPC-treated water (0.1% diethyl pyrocarbonate). Electrophoresis tanks were washed with detergent, rinsed in water and then incubated in 3% H_2O_2 for 10 min at RT before giving a final rinse with water.

2.10.2 RNA Isolation from *L. lactis* cells

RNA isolation from *L. lactis* was performed by the hot phenol method (Magni *et al.*, 1995) with modifications (Lubbers *et al.*, 1998). 40 ml of GM17 medium was inoculated with 500 µl *L. lactis* MG1363 ON culture and grown to an OD_{600} of 0.7, the cells pelleted at 3,000 rpm for 10 min at 4°C using a SS34 rotor (RC-5B, Sorvall centrifuge), and the pellet frozen at -80°C. The cells were resuspended on ice in 2 ml buffer (1% (w/v) SDS, 0.9 mM EDTA, 18 mM sodium acetate, pH 5.5) and transferred to 2 ml of hot water-saturated phenol-chloroform (70°C). The tubes were then incubated at 70°C for 10 min with periodical vortexing and then centrifuged at 4,000 rpm for 2 min. The supernatant was transferred to 2 ml of hot (70°C) water-saturated phenol (pH 4.5), vortexed periodically and centrifuged as above; the hot phenol extraction was performed twice. After extraction, the supernatant was transferred to 2 ml of chloroform, vortexed 2-3 times and centrifuged as in the previous step. The supernatant was transferred to a new tube and the RNA precipitated with 0.1 vol 3 M sodium acetate (pH 7.0) and 2.5 vol 100% ethanol at -80°C for 30 min. The tube was then centrifuged at 10,000 rpm for 15 min at 4°C using the SS34 rotor, and the pellet washed briefly with 70% ethanol and resuspended in 100 µl 1x TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.5).

The resuspended RNA was extracted twice with 1 vol Tris buffered phenol (pH 8), followed by one extraction with 1 vol Tris-buffered phenol-chloroform (pH 8) and one extraction with 1 vol chloroform. The RNA was precipitated again with 0.1 vol 3 M sodium acetate (pH 7.0) and 2.5 vol 100% ethanol for 30 min at -80°C. After centrifugation at 14,000 rpm (20,800 xg) for 20 min at 4°C (Eppendorf centrifuge), the pellet was washed briefly with 70% ethanol and resuspended in 100 µl 1x TE buffer (pH 7.5). The RNA was treated with DNase (1 U DNase I/µg RNA (Invitrogen, Carlsbad, USA), 20 U RNaseOUT™ (Invitrogen, Carlsbad, USA), 7 mM $MgCl_2$, 0.7 mM DTT) for 45 min at RT. 0.01 vol of 200 mM EDTA was then added and the RNA extracted with 1 vol Tris-buffered phenol-chloroform and 1 vol chloroform. The RNA was reprecipitated with 0.1 vol 3 M sodium acetate (pH 7.0) and 2.5 vol 100% ethanol and pelleted by centrifugation in an Eppendorf centrifuge at 14,000 rpm (20,800 xg) for 20 min at 4°C. The RNA was then washed briefly in 70% ethanol and resuspended in 50 µl 1x TE buffer (pH 7.5) before storage at -80°C.

2.10.3 RNA analysis methods

2.10.3.1 RNA agarose gel electrophoresis

RNA was separated and visualized on horizontal agarose gels (Horizon ® 58 mini gel apparatus, Invitrogen, Carlsbad, USA). Before using the gel box for separation of RNA it was thoroughly cleaned with 3% H_2O_2 and RNase*AWAY*™ (Molecular Bio-Products, San Diego, USA). 2% (w/v) agarose gels were prepared with 1x MOPS buffer (20 mM 3-(N-morpholino) propane-sulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). The agarose was dissolved in the buffer by microwaving, and the mixture cooled to 55°C. Immediately after cooling, formaldehyde was added to the gel mixture (5% final concentration) and the gel cast. The RNA samples were mixed with an equal volume of RNA loading buffer (50% formamide, 5% glycerol, 16.7% formaldehyde, 1x MOPS, 0.4 mg/ml bromophenol blue), heated to 65°C for 15 min and then stored on ice for 10 min. 2 µl of 500 µg/ml ethidium bromide were added to the samples before loading on the gel and the gel run at 40 V for 2-3 hours in 1x MOPS buffer.

The RNA was visualized under UV irradiation using the TMW-20 Transilluminator (Alpha Innotech), and the images captured by the Alpha Imager 2000 System (Alpha Innotech). The standard RNA molecular weight markers (0.24-9.5 kb RNA ladder, Invitrogen, Carlsbad, USA; 0.16-1.77 kb RNA ladder, Invitrogen, Carlsbad, USA) were treated in the same way as the RNA samples.

2.10.3.2 RNA polyacrylamide gel electrophoresis

Denaturing polyacrylamide gel electrophoresis (PAGE) was used for the separation of low molecular weight RNA (100-500 nt). 6 M urea/6% polyacrylamide gels (5.4 g urea, 1.5 ml 10x TBE, 3 ml 30% acrylamide/bis acrylamide (19:1), 120 µl of 10% APS, 16 µl TEMED, total volume 15 ml) were run in a Mini-Protean ® II Electrophoresis Cell (Bio-Rad). The RNA samples (10-20 µg total RNA) were mixed with loading dye (80% deionized formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) and incubated at 75°C for 10 min. After cooling for 5 min on ice, the samples were loaded onto the gel and PAGE electrophoresis was performed at 100 V for 40-50 min in 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). A low molecular weight RNA ladder was used as a standard (0.16-1.77 kb RNA ladder, Invitrogen, Carlsbad, USA).

2.10.3.3 RNA quantification

The concentration of RNA was determined spectrophotometrically, using essentially the same method as was used for measuring DNA concentrations (section 2.7.3). The RNA concentration [µg/ml] was calculated by multiplying the absorbance reading at 260 nm by a constant factor (40 for ssRNA) and the dilution factor. The purity of the RNA sample was evaluated by the ratio of the absorbance readings at 260 nm and 280 nm reading. Pure RNA samples generated a ratio ≥2.0, while samples with significant protein contamination generated ratios lower than 2.0.

2.10.3.4 Northern blotting

RNA samples were separated according to their molecular mass by RNA agarose gel electrophoresis. The gel was washed twice for 5 min in water, and then twice for 20 min in 20x SSC (3 M NaCl, 0.3 M sodium citrate). An ON capillary nucleic acid transfer to positively charged nylon membrane (Roche, Mannheim, Germany) was performed following the standard procedure (Sambrook *et al.*, 1989). The RNA was fixed to the membrane by UV-crosslinking for 2 min using the TMW-20 Transilluminator (Alpha Innotech).

RNA separated by denaturing PAGE (section 2.10.3.2) was electrotransferred (Bio-Rad Mini Trans-Blot) from the gel onto a positively charged nylon membrane (Roche, Mannheim, Germany). The transfer was performed for one hour at 300 mA using 1x TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) as the transfer buffer. The RNA was fixed to the membrane by UV-crosslinking for 2 min using the TMW-20 Transilluminator (Alpha Innotech) and the RNA transfer visualized by methylene blue staining of the blot (Herrin and Schmidt, 1988). The blot was stained (0.3 M sodium acetate, pH 5.2, 0.03% methylene blue) for 2-5 min, destained in 0.5% SDS for 3 min and washed for 2-5 min in water.

2.10.3.5 Northern hybridization using ECL™-labelled probes

Transcripts of interest were detected by Northern blotting; the RNA was hybridized with ECL™ (enhanced chemiluminescence, Amersham Pharmacia Biotech, Little Chalfont, England) labelled probes and detected by chemiluminescence. Labelling of the DNA probes, hybridization and detection were performed according to manufacturer's instructions. DNA probes were prepared by PCR and diluted to a concentration of 10 ng/µl. 100 ng of the probe was then denatured by heating for 5 min at 100°C, and the DNA cooled immediately on ice for 5 min. The DNA was mixed gently with an equal volume of DNA labelling reagent (positively charged complexes of peroxidase) and crosslinking between the probe and peroxidase initiated by the addition of 10 µl glutaraldehyde solution (10 min at 37°C).

The blot was pre-hybridized in ECL™ hybridization buffer (hybridization buffer, Amersham Pharmacia Biotech, Little Chalfont, England, supplemented with 0.5 M NaCl, and 5% blocking reagent) for one hour at 42°C and the labelled probe added. Hybridization between the probe and the immobilized RNA fragments was performed ON at 42°C in a hybridization oven (Bachofer). Washes were performed according to the manufacturer's instructions (two 20 min washes at 42°C in 6 M urea, 0.4% SDS, 0.5x SSC (saline-sodium citrate buffer), followed by two washes for 5 min at RT in 2x SSC).

The bound probe was detected by mixing equal volumes of detection reagent 1 and 2 (normally 1.5 ml each) and adding them directly to the probe-side of the blot. Detection Reagent 1 decays to hydrogen peroxide, the substrate for peroxidase. Upon contact with the probe, hydrogen peroxide becomes reduced and luminol (Detection Reagent 2) oxidized by the peroxidase. The oxidized luminol emits a blue light, which can be detected on a blue-light sensitive film. The blot was incubated for 1 min at RT and the excess detection reagent drained off. The blot was then transferred into a plastic bag, placed DNA side up in a film cassette and exposed to x-ray film (Fuji Super RX) for up to 10 min. The film was developed using an automatic x-ray film processor (100 Plus™, All Pro Imaging).

2.10.3.6 Transcript analysis by primer extension

Primer extension analysis was used to determine the location of the 5'-end (transcription start site) of RNA. The AMV Reverse Transcriptase Primer Extension System (Promega, Madison, USA) was used according to the manufacturer's instructions. An end-labelled oligonucleotide was hybridized to RNA and then utilized as a primer by reverse transcriptase in the presence of deoxynucleotides. The synthesized cDNA was analyzed on a denaturing polyacrylamide gel. A sequencing reaction of the equivalent DNA region using the same end-labelled primer was used as the size marker. The length of cDNA equals the number of bases between the 3' end of the primer and the 5' end of the RNA.

The primer extension analysis was performed as follows. A primer for the analysis was designed to anneal downstream of the putative transcription start site. 10 pmol of the PAGE-purified primer was end-labelled using T4 polynucleotide kinase and 30 μCi [γ-^{32}P] ATP (Amersham Pharmacia Biotech, Little Chalfont, England). 100 fmol end-labelled primer and 20 μg total RNA isolated from *L. lactis* were used for cDNA synthesis. The primer was annealed at 58°C for 90 min, extension performed at 42°C for 30 min, and the products from the primer extension reaction analyzed on a conventional sequencing gel (section 2.7.4).

2.10.3.7 Transcript analysis by electrophoretic gel migration shift assay (EMSA)

To determine if there were any proteins binding to the transcripts, EMSA assays were performed using radiolabelled sense RNA probe (P_E1 transcript) and total lactococcal cell extract. An excess of tRNA was used to prevent non-specific RNA-binding proteins from binding to the probe. In this step, 20 μg lactococcal cytoplasmic cell extract was incubated with 10 μg tRNA at 4°C for 20 min in binding buffer (0.1 mM EDTA, 0.2 mM DTT, 8 mM $MgCl_2$, 4 mM spermidine, 3 mM ATP, 10% glycerol, 10 mM HEPES, pH 7.4). 1-2 ng of ^{32}P-labelled sense probe (Riboprobe system T7, Promega, Madison, USA) was added to give a final volume of 10 μl, and the reaction mixtures incubated at 4°C for an additional 30 min. The sense probe used for the EMSA corresponded either to the 307 nt or 207 nt transcript synthesized from P_E1. For competition assays, unlabelled competitor RNA was included as a pre-incubation step. RNA-protein complexes were resolved by electrophoresis on a 6% nondenaturing polyacrylamide gel using 0.5x TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) as the running buffer. The gels were pre-run for 30 min at 10 mA at RT. After adding 2 μl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol) to the samples, the samples were loaded onto the gel, and the gel run for 3-4 hours using the same conditions as in the pre-run. The gel was then fixed in 10% acetic acid, dried and autoradiographed.

2.10.3.8 Transcript analysis by RNase protection

To determine the exact length of the transcripts, RNase protection experiments were performed. Briefly, radiolabelled antisense RNA probes with defined endpoints were produced by *in vitro* transcription and hybridized to the target RNA. RNase was added to the hybridization mixture, thus only ssRNA was digested, while the RNA:RNA hybrids remained. After proteinase inactivation of RNase, the RNA was purified by phenol extraction and precipitated. The protected probe fragments were then separated using PAGE and their size determined by comparison to a ladder.

A detailed description of the RNase protection analysis is given below. The DNA sequence coding for the P_E1 transcript was cloned into pGEM®-3Zf$^-$ (resulting in pGEMc2, Rakonjac *et al.*, 2002, submitted) and the plasmid linearized by restriction with *Bam*HI. The riboprobe system T7 (Promega, Madison, USA) was used for *in vitro* transcription of pGEMc2 with [α-^{32}P] CTP to produce "antisense" RNA probe. Gel loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 mM EDTA) was added to the radiolabelled transcript, heated for 10 min at 75°C, and then run on a 5% polyacrylamide gel containing 8 M urea (400 V) using 1x TBE as a running buffer. The RNA was excised from the gel and eluted ON in buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.2% (w/v) SDS) at 37°C. The RNA was then precipitated (2.5 vol 100% ethanol, 0.1 vol 3 M ammonium acetate and 20 µg tRNA) and resuspended in RNase-free water. 10-15 µg total lactococcal RNA was mixed with 10^5 cpm of the antisense probe, heated to 85°C for 5 min in 40 µl hybridization buffer (50% deionized formamide, 400 mM NaCl, 1 mM EDTA, 40 mM PIPES, pH 6.7), and then hybridized ON at 37°C. The RNA:RNA hybrid was digested with 1-10 U RNase ONE™ (Promega, Madison, USA) in 300 µl digestion buffer (5 mM EDTA, 200 mM sodium acetate, 10 mM Tris-HCl, pH 7.5) at RT for 20 min. The reaction was terminated with 20 µl of 10% SDS and 50 µl of 1 mg/ml proteinase K, and the RNA extracted with 1 vol phenol-chloroform (pH>8), then precipitated with 2.5 vol 100% ethanol using 2 µg tRNA as a carrier. The RNA was resuspended in gel loading buffer (section 2.10.3.2), heated to 85°C for 5 min and then separated by denturing PAGE (5% polyacrylamide/8 M urea (7.2 g urea, 1.5 ml 10x TBE, 2.5 ml of 30% acrylamide/ bis acrylamide (19:1), 120 µl of 10% APS, 16 µl TEMED, 15 ml total volume) using 1x

TBE as a running buffer. The protected, labelled antisense RNA was then detected by autoradiography.

To determine the length of the protected fragments, the following sets of standards were used: ^{32}P-end-labelled low range RNA (0.16-1.77 kb) ladder, ^{32}P-end-labelled 1 kb Plus ladder (both Invitrogen, Carlsbad, USA) and a ^{32}P-labelled sequencing reaction.

2.10.3.9 Transcript analysis by secondary structure modelling

Computer programs were used to predict the possible secondary structures and pseudoknots of the transcripts synthesized from P_E1, using the lowest free energy of formation (ΔG). The prediction of RNA folding is based on free energy minimization using the nearest neighbour parameter of Turner (Xia *et al.*, 1998). The mfold program uses the Zuker algorithm to predict secondary structures (Mathews *et al.*, 1999) and then re-orders the favourable structure using a second algorithm (efn2).

The P_E1 transcript structure was modelled using mfold, (http://www.ibs.wustl.edu/~zuker; Mathews *et al.*, 1999); RNAfold (http://www.tbi.univie.ac.at/cgi-bin/RNAfold.cgi; Walter *et al.*, 1994; Mathews *et al.*, 1999), RNAstructure (http://128.151.176.70/RNAstructure.html; Mathews *et al.*, 1999) and RNAdraw (http://rnadraw.base8.se/#wwwRnadrawSysReqInst; Matzura and Wennborg, 1996). The PKnots program was used to search for pseudoknot forming sequences (http://www.genetic.wustl.edu/eddy/software/#pk; Rivas and Eddy, 1999), and the program RNAviz was used to redraw RNA structures (http://rrna.uia.ac.be/rnaviz/; de Rijk and de Wachter, 1997).

2.11 Protein preparation

2.11.1 Cytoplasmic cell extract preparation

Cytoplasmic proteins of *L. lactis* MG1363 were prepared using the French-press. 800 ml of GM17 medium was inoculated with 1 ml *L. lactis* MG1363 ON culture and grown to an OD_{600} of 0.4. The cells were pelleted (centrifugation at 3,000 rpm for 10 min at 4°C using a GSA rotor), washed in 0.9% NaCl, recentrifuged (as above) and resuspended in 5 ml resuspension buffer (70 mM KCl, 10% glycerol, 1 mM EDTA, 7 mM $MgCl_2$, 1 mM β-mercaptoethanol, 50 mM Tris-HCl, pH 7.4). The resuspended culture was forced through a French press cell three times (7,500 psi) to break the cells and the cellular debris removed by centrifugation at 13,000 rpm for 15 min at 4°C using a SS34 rotor (RC-5B Sorvall centrifuge). The supernatant was transferred to Eppendorf tubes and stored at -80°C.

2.11.2 Protein analysis

2.11.2.1 Protein electrophoresis

Proteins were separated according to their molecular weight by vertical electrophoresis using denaturing SDS polyacrylamide gels (SDS-PAGE; Laemmli, 1970). Electrophoresis was performed using the Mini-Protean ® II Electrophoresis Cell (Bio-Rad). The gels were composed of a 12% separating and a 5% stacking gel. The separating gel was a mixture of 12% acrylamide-bisacrylamide (37.5:1, Bio-Rad), 0.375 M Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.1% (w/v) freshly prepared ammonium persulfate (APS) solution and 0.04% TEMED. The separating gel was cast and overlaid with a mixture of isobutanol and water. During polymerization of the separating gel, the stacking gel was prepared (5% acrylamide-bisacrylamide (37.5:1), 0.13 M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.1% (w/v) freshly prepared APS, 0.1% TEMED). The isobutanol layer was thoroughly rinsed from the top of the separating gel and the stacking gel cast.

Protein samples were mixed with an equal vol of final sample buffer (10% (w/v) glycerol, 3% (w/v) SDS, 0.01% (w/v) bromophenol blue, 62.5 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol added immediately before use), boiled for 5 min and stored on ice. The gel was run for 45-50 min at 20 mA in running buffer (25 mM Tris-HCl, 250 mM glycine, 0.1% (w/v) SDS, pH 8.3). After electrophoresis, the gel was stained in Coomassie Brilliant Blue R 250 stain (45.5% methanol, 9.2% acetic acid, 0.25% (w/v) Coomassie Brilliant Blue R 250) for 30 min at RT.

The gel was destained in several changes of a mixture of 45.5% methanol and 9.2% acetic acid until the background coloration was low. To determine the molecular weight of the sample proteins, a pre-stained molecular weight marker (Invitrogen, Carlsbad, USA) was run next to the samples.

If the protein bands detected with the Coomassie stain were very weak, silver stain was used instead. The silver stain method is described below. The gel was soaked for 10 min in 50% methanol, 10 min in 0.032 M DTT, and for 10 min in 0.1% $AgNO_3$. After rinsing three times in water, the gel was immersed in developer (0.06% formaldehyde, 0.032 M Na_2CO_3) until the protein bands were visible. The development process was terminated by fixing the gel in 2% acetic acid (10 min) and then soaking the gel in water.

2.11.2.2 Protein quantification

Protein concentrations were determined using the Bio-Rad protein assay according to the manufacturer's instructions. The assay is based on the method of Bradford (1976) and involves the addition of an acidic dye (Coomassie ® Brilliant Blue G-250) to the protein solution and subsequent measurement of the mixture at 595 nm with a spectrophotometer. The dye binds to the proteins in the solution with the color change increasing in direct proportion to the protein concentration.

A six point standard curve was prepared using BSA (bovine serum albumin, range of 0 to 10.0 µg/ml). 800 µl of each standard and 800 µl of each sample were transferred to Eppendorf tubes, 200 µl of the dye reagent concentrate added, and the tubes vortexed and incubated at RT for a minimum of 5 min.

The absorbance was measured at 595 nm (Nova Tech spectrophotometer) and all standards and samples were assayed in triplicates. Protein concentration of the samples were calculated by interpolation from the BSA standard curve.

2.11.2.3 North-Western blot

North-Western blot analysis was used to identify the putative P_E1 transcript binding proteins. Lactococcal cell extract proteins were separated using 12% SDS polyacrylamide gels, and transferred by electroblotting onto a 0.2 μm nitrocellulose membrane (Sartorius) using a mini Trans-Blot apparatus (Bio-Rad). The electrotransfer was performed for one hour at 30 V using glycine transfer buffer (192 mM glycine, 20% methanol, 25 mM Tris, pH 8.3). The membrane-bound proteins were renatured by incubating the membrane in renaturation buffer (1 mM EDTA, 50 mM NaCl, 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.5, and 1x Denhardt reagent (0.02% (w/v) Ficoll type 400, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) BSA)) at RT for 1 hour with gentle agitation. The renaturation step was repeated three more times using fresh renaturation buffer. The membrane was then incubated at RT for 30 min in renaturation buffer containing 20 μg tRNA. Transcript-binding proteins were detected by adding [α-^{32}P] CTP radiolabelled transcript (*in vitro* transcription, Riboprobe system T7, Promega, Madison, USA) to the renaturation buffer and incubating the mixture for 1.5 hours at RT. The membrane was then washed (three times 15 min each) in renaturation buffer at RT, air-dried and exposed to x-ray film (Fuji Super RX). A control transcript (T7 gene 10, Riboprobe T7 *in vitro* transcription kit, Promega) was included in the assay.

2.11.2.4 Affinity purification of RNA-binding proteins

The lactococcal proteins binding to the transcript were purified as described below. Biotinylated RNA was attached to streptavidin-coated magnetic particles, and the particles incubated in lactococcal cell extract. The RNA-bound proteins were then affinity purified by magnetic separation and eluted using a high salt buffer (Gabrielsen *et al.*, 1989).

In detail, biotinylated RNA was synthesized with the MEGA script transcription kit (Ambion) using biotin-16-UTP (Roche, Mannheim, Germany) at a biotin-16-UTP/UTP ratio of 3.5:6.5. 400 µl of streptavidin-coated magnetic particles (Roche, Mannheim, Germany) were washed three times at RT in TEN_{100} (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5).

The beads were then incubated with 30 µg biotinylated RNA in the same buffer for 20 min at RT. After magnetic separation, unbound RNA was removed by washing in 4 ml of TEN_{1000} (10 mM Tris-HCl, 1 mM EDTA, 1 M NaCl, pH 7.5), and the beads resuspended in binding buffer (1 mM EDTA, 15% glycerol, 0.05% Nonidet, 120 mM KCl, 15 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.0). 80 µg cytoplasmic cell extract was then pre-incubated with 80 µg tRNA for 15 min on ice and the mixture added to the beads. The beads were then rotated at 4°C for 30 min, and washed four times in binding buffer. Protein bound to the biotinylated RNA was eluted with binding buffer containing 1 M KCl, and the eluted proteins separated by SDS-PAGE (12%). Protein detection was either by Coomassie blue staining (section 2.11.2.1), silver staining (section 2.11.2.1) or North-Western blotting (section 2.11.2.3).

2.11.2.5 Amino-terminal sequencing of RNA-binding proteins

To obtain sufficient quantity of protein for amino-terminal amino acid sequencing, preparative scale affinity purification of the RNA-binding proteins was performed. Eluted proteins were separated by SDS-PAGE (12%) and transferred to PVDF protein membranes (Millipore) using a Bio-Rad Mini Trans-Blot® electrophoretic transfer cell. The transfer was performed at 30 V for one hour at RT using CAPS transfer buffer (10 mM CAPS, 10% methanol, pH 11.0; LeGendre and Matsudaira, 1988). The blot was stained for 5 min in Coomassie blue R-250 (0.1% (w/v) in 50% methanol) and destained in several changes of a mixture of 50% methanol and 10% acetic acid. Relevant bands were excised from the membrane and their N-terminal sequences determined by Edman degradation. Sequencing was performed using a PE Biosystem protein sequencer (Model 476A) at the Massey University Protein Sequencing Facility (Palmerston North).

Protein databases were searched for matches to the N-terminal sequences of the transcript binding proteins. Database searches for translated sequences were performed on the server of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and database searches for translated *L. lactis* sequences were performed on the INRA (Institute National de la Recherche Agronomique, http://www.inra.fr) server. Multiple sequence alignments were created with the Clustal W program (Thompson *et al.*, 1994) (http://www.ebi.ac.uk/clustalw).

2.12 Statistical analysis of variance between populations

2.12.1 The ANOVA procedure

The different growth curves and growth rates for *L. lactis* containing the various plasmids were determined (section 3.5.2) and compared. To investigate if differences in the growth rates were statistically significant, the ANOVA procedure (analysis of variance) was applied. Thus the variability between the growth rates (s_B^2) and the variability within the growth rates (s_w^2) was measured (standard deviations). For k samples of size n_1, n_2, ...n_k, the *F* test was used to verify the equality of population means.

$$F = \frac{{s_B}^2}{{s_W}^2}$$

where

$${s_W}^2 = \frac{(n_1 - 1){s_1}^2 + (n_2 - 1){s_2}^2 \cdots + (n_k - 1){s_k}^2}{n_1 + n_2 + \cdots + n_k - k}$$

and

$${s_B}^2 = \frac{\sum n_i \bar{y}_i^2 - (\sum n_i \bar{y}_i)^2 / n}{k - 1}$$

s_i^2 = variance of sample *i*
n_1 = size of sample *i*
k = number of samples
n = Σn_i
$\overline{y}_i$ = mean of sample *i*

If the null hypothesis H_0 (μ_1, μ_2, ...μ_k, equality of the population means) is true, then *F* is expected to assume a value near *F* = 1. If the null hypothesis H_0 is false, the null hypothesis H_0 is rejected in the upper tail of the distribution of $F = s_B^2/s_W^2$.
H_o is rejected if $F > F_{\alpha, df1, df2}$ where df1 = (k-1) and $df_2 = (n_1 + n_2 + ... + n_k - k)$ and α = probability of type I error.

To analyze the data by computer, the ANOVA procedure of the Statistical Analysis System (SAS) by the SAS Institute Inc., NC, USA (Version 8.02) was used.

3. RESULTS

3.1 Modifications of the P_E1 promoter

Waterfield *et al.* (1996) have shown that the cloned intergenic region of phage c2 supports plasmid replication. The c2 *ori*-plasmid pLP201 (Waterfield *et al.*, 1996, previously named pVA891-*ori*) contains P_E1 (early promoter 1) and P_L1 (late promoter 1), and 307 bp of the non-coding region downstream of P_E1 (Figure 3.1). This c2-*ori* fragment supports replication of the lactococcal origin screening vector pVA891 (Waterfield *et al.*, 1996) in *L. lactis* (Table 3.1). The plasmid pVA891 cannot replicate in *L. lactis* because it lacks an origin of replication for Gram-positive bacteria (Macrina *et al.*, 1983). Therefore, the cloned c2 fragment must contain a functional origin of replication. Gordon (1997) investigated if P_L1 and the highly conserved AT-rich region between P_E1 and P_L1 play any role in replication by deleting these elements. The remaining fragment, which contained P_E1 and the 307 bp non-coding sequence downstream of P_E1, was cloned into pVA891 to generate pLP203 (Figure 3.1). Gordon (1997) found that the shortened *ori* fragment in pLP203 still supported plasmid replication in *L. lactis* (Table 3.1) and these results suggest that P_E1 and the sequence downstream of P_E1 are sufficient for origin function, and that P_L1 and the conserved AT-rich region are not required for replication. The sequence downstream of P_E1 is very poorly conserved in prolate phages (Waterfield *et al.*, 1996; Rakonjac *et al.*, 2003), and is transcribed from P_E1 during phage c2 infection (Lubbers *et al.*, 1998).

The requirement for a functional promoter for replication, was investigated by creating several c2 *ori* fragments with modified P_E1 promoters and cloning them into pVA891. Two T to G substitutions in the consensus -10 region of P_E1 (TATAAT to TAGAAG) were constructed by using a PCR based strategy (pLP205, Callanan, unpublished results, Figure 3.1). The substitutions were designed to eliminate or significantly reduce P_E1 promoter activity with minimal impact on DNA conformation in the origin. On the basis of a limited number of electroporation trials it was tentatively suggested that this plasmid does not replicate in *L. lactis,* and that the two point mutations in P_E1 abolished replication. However in the current study, repeated

electroporation of pLP205 into *L. lactis* MG1363 showed that this plasmid is able to replicate in *L. lactis* and that the c2 *ori*-fragment in pLP205 supported plasmid replication despite the modified P_E1 promoter (Table 3.1). Thus, the previous results were shown to be an experimental artifact.

The requirement for a functional promoter for replication was further investigated by deleting the whole P_E1 promoter (-10 and -35 hexamer sequence, and spacer sequence) using a PCR-based strategy (Gordon, 1997; Figure 3.1). The new plasmid pLP204 was unable to replicate in *L. lactis* MG1363. This conclusion was based on the repeated and reproducible failure to electroporate pLP204 successfully into *L. lactis* MG1363 compared to parallel controls (Table 3.1).

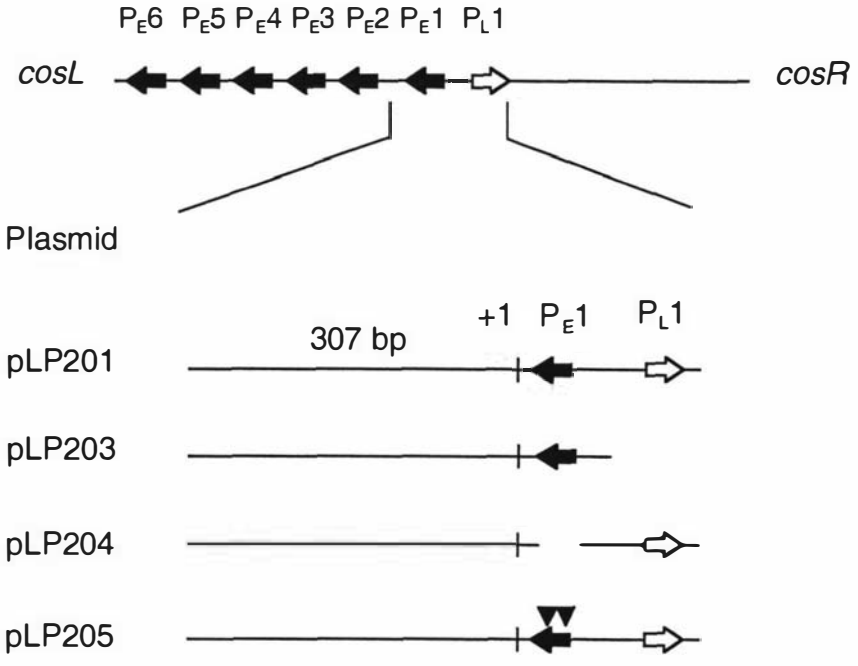


Figure 3.1. Schematic representation of the c2 genome and the c2 *ori*-plasmids pLP201, pLP203, pLP204 and pLP205.

The c2 *ori*-plasmids with modified, deleted or inverted P_E1 promoters and deleted P_L1 promoter are shown. The plasmids were made by M. Lubbers (Waterfield *et al.*, 1996), J. Gordon (Gordon, 1997) and M. Callanan (Callanan, unpublished results). The black arrows (⬅) represent the early promoter 1 (P_E1), and the white arrows (⇨) represent the late promoter 1 (P_L1). In pLP204, P_E1 is deleted, and the two black triangles (▼▼) in pLP205 represent T to G substitutions.

Table 3.1. Transformation frequencies of *ori*-plasmids with modified P_E1 promoters for *L. lactis* MG1363.

Plasmid	Average electroporation frequency of *L. lactis* MG1363 [cfu/μg][1] (S.D.)
pLP201	$2.7\pm0.2 \times 10^3$
pLP203	$4.6\pm0.7 \times 10^4$
pLP204	<2
pLP205	$8.5\pm0.6 \times 10^3$
pLP213	<2
water	<2

[1]Electroporation frequency was measured as the number of ery^R colonies per μg of DNA used in transformation. The data shown represent the average of three independent experiments. 500 ng of DNA was used for each electroporation. There was a slight variation in transformability between the batches of electrocompetent cells used in each experiment.

3.1.1 Replacement of P_E1 with the *nisA* promoter

In order to distinguish whether it is the P_E1 promoter activity or its sequence which is required for c2-*ori* replication, Callanan (unpublished results) replaced P_E1 with the inducible *nisA* promoter (Dodd *et al.*, 1990; de Ruyter *et al.*, 1996a) (pLP212, Figure 3.2). It has previously been published that in the absence of nisin, the *nisA* promoter is switched off and nisin is needed for promoter induction (Kuipers *et al.*, 1995). In addition to nisin, two other proteins have been implicated in the regulation of transcription from *nisA*. In the presence of extracellular nisin, the membrane located NisK (histidine kinase) is autophosphorylated. Autophosphorylation of NisK leads to the subsequent phosphorylation of NisR, which in turn activates the *nisA* and *nisF* promoters.

Callanan (unpublished results) constructed a plasmid in which the P_E1 promoter was replaced by the inducible nisin promoter. This was achieved by deleting the P_E1 promoter in the pUC19-*ori* plasmid by inverse PCR, amplifying the *nisA* promoter by PCR (template pNZ8037, de Ruyter *et al.*, 1996b), ligating the two fragments, and then recloning the new *ori*-fragment containing the *nisA* promoter into the *Eco*RI site of pVA891 (Figure 3.2). In the present study, the ability of this new plasmid (pLP212) to replicate in *L. lactis* was tested by electroporation into *L. lactis* strains NZ9000 and

NZ9800 (NIZO Food Research, Ede, Holland) in the presence and absence of nisin inducer (as Nisaplin; Aplin & Berrett, Beaminster, United Kingdom). These strains were used because they both contain the *nisR* and *nisK* genes, which are involved in regulation of nisin induction, but they do not produce the nisin peptide (Kuipers *et al.*, 1993; Kuipers *et al.*, 1998). Nisaplin was used for the induction of the *nisA* promoter (commercially available form of nisin).

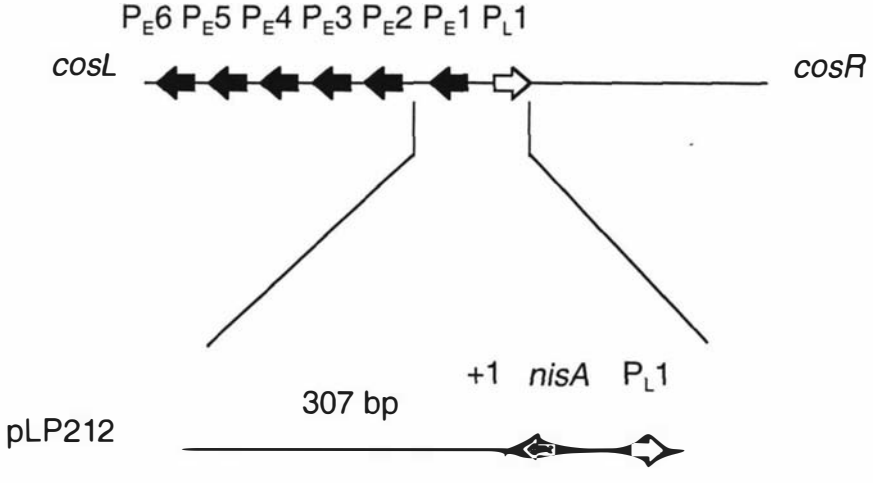


Figure 3.2. Schematic representation of the c2 genome and the c2 *ori*-plasmid pLP212.

The P_E1 promoter is replaced by the inducible *nisA* promoter, represented by a gray arrow (⇦). The white arrow (⇨) represents the late promoter 1 (P_L1), and the black arrows (⬅) represent the early promoters. The plasmid was made by M. Callanan (Callanan, unpublished results).

The plasmid pLP212 was successfully introduced by electroporation into both strains (Table 3.2). The electroporation experiment was performed three times, and water instead of DNA was used in the negative control. Surprisingly the plasmid could be introduced even in the absence of nisin (Table 3.2), the conditions under which the *nisA* promoter should be repressed (Kuipers *et al.*, 1998). However, in the presence of nisin, the transformation frequency was one to two orders of magnitude higher than in the absence of nisin.

Table 3.2. Transformation frequencies of pLP212 for *L. lactis* NZ9000 and NZ9800.

Plasmid pLP212	**Average electroporation frequency of *L. lactis* [cfu/μg][1] (S.D.)**	
	NZ9000	**NZ9800**
without induction of nisaplin	$2.3\pm0.5 \times 10^2$	$1.6\pm0.5 \times 10^2$
150 ng ml^{-1} nisaplin	$1.9\pm1 \times 10^4$	$1.7\pm0.1 \times 10^3$
water	<2	<2

[1]Electroporation frequency was measured as the number of ery^R colonies per μg of DNA used in transformation. The data shown represent the average from three independent experiments. 500 ng of DNA was used for each electroporation. There was a slight variation in transformability between the batches of electrocompetent cells used in each experiment.

3.1.2 Inversion of P_E1

Loss of *ori* function by deletion of P_E1 in pLP204 could have been due to the lack of a transcript with a necessary mechanistic role, or because of DNA conformational changes in the *ori* fragment caused by the internal deletion. To help distinguish between these two possibilities, P_E1 was inverted in the plasmid pLP203 to generate pLP213 (Figure 3.3). The inversion was produced using inverse PCR on pUC19-203 with primers AS1 and AS2, which contained each 14 bp of the P_E1 promoter sequence in an inverted orientation at their 5' ends. The PCR product was gel purified, phosphorylated, self-ligated and then *E. coli* ER2206 cells were transformed using the ligation product and the cells selected for ampicillin resistance. Self-ligation of the PCR product joined the P_E1 promoter sequence elements together. After sequencing the insert, the *ori* fragment containing the inverted P_E1 promoter was excised with *Eco*RI, gel purified and subsequently cloned into the *Eco*RI site of pVA891. The new plasmid pLP213 (Figure 3.3) was sequenced (primer LX-1) to confirm that no base pair changes had occurred in the c2 *ori* insert.

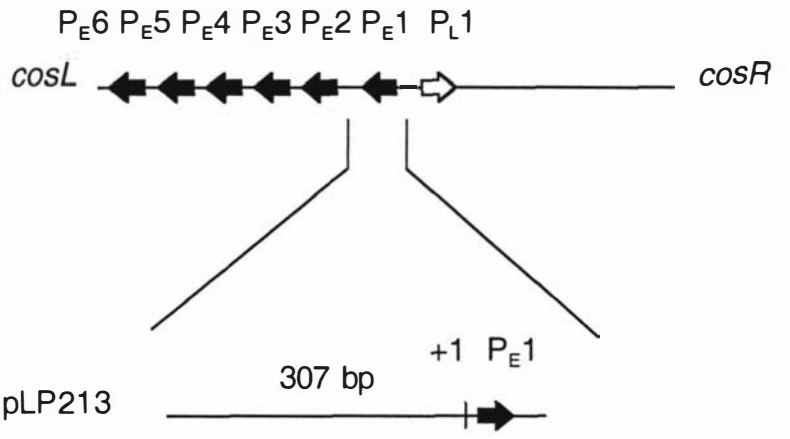


Figure 3.3. Schematic representation of the c2 genome and the c2 *ori*-plasmid pLP213.

In plasmid pLP213, P_E1 is inverted. The black arrows (⬅) represent the early promoters, the white arrow (⇨) represents the late promoter.

To investigate whether the *ori*-fragment with the inverted P_E1 promoter could support plasmid replication, pLP213 was electroporated into *L. lactis* MG1363. As a positive control, *L. lactis* MG1363 cells were also electroporated with pLP201 and pLP205, and in the negative control the DNA was replaced with water. Although the electroporation experiment was performed three times, no transformants could be recovered for pLP213 (Table 3.1). Since pLP213 contains the active P_E1 promoter in reverse orientation, these results suggested that the events of RNA polymerase binding to the promoter of the *ori* fragment and the opening of the DNA strands during transcription were not sufficient for replication. Thus it can be concluded that transcription of the sequence downstream of P_E1 is probably also required for replication. It was attempted to transform *L. lactis* cells with pLP213 and pTRKL2 (as a control) at the same time, however the plasmids were incompatible.

3.2 Alterations of the transcript synthesized from P_E1

3.2.1 Deletion analysis of the DNA sequence coding for the transcript synthesized from P_E1

Waterfield *et al.* (1996) showed that a 521 bp DNA fragment from the c2 origin, containing P_L1, P_E1 and the region downstream of P_E1, cloned into pVA891, supported plasmid replication of pVA891 in *L. lactis*. However, a 261 bp fragment containing P_L1, P_E1 and a 48 bp DNA sequence coding for the P_E1 transcript (starting from the +1 nucleotide) did not support plasmid replication of pVA891 in *L. lactis* (Waterfield *et al.*, 1996). This result suggests that the transcript has to be a certain length to support pVA891 replication. Therefore one aim of this project was to identify the length of the P_E1 transcript required for replication.

Gordon (1997) had already shown that P_L1 is not required for the c2 *ori* fragment to support plasmid replication (pLP203, Figure 3.1). Therefore all new *ori* fragments constructed in the present study, contained P_E1 and the sequence downstream of P_E1, but not P_L1. PCR primers were designed to create a series of six c2 *ori* constructs. This was achieved by amplifying the c2 *ori* and thereby progressively shortening the DNA sequence coding for the P_E1 transcript from the 3' end (primers LP206, LP207, LP208, LP209, LP210 and LP211 each in combination with the latedel primer; template pLP203). After amplifying the c2 *ori* fragments by PCR, the products were digested with *Eco*RI, gel purified and then cloned into the *Eco*RI site of pUC19. The six fragments were verified by DNA sequencing, and the *ori* inserts recloned into the *Eco*RI site of pVA891 resulting in pLP206-pLP211 (Figure 3.4). Cloning the *ori* fragments into the *Eco*RI site of pVA891 disrupts the chloramphenicol resistance gene of pVA891. Therefore, cells containing pVA891 with the c2 *ori* inserts were chloramphenicol sensitive and erythromycin resistant. All of the inserts of the new constructs were sequenced (primer LX-2) to ensure that no base pair changes in the sequence had occurred.

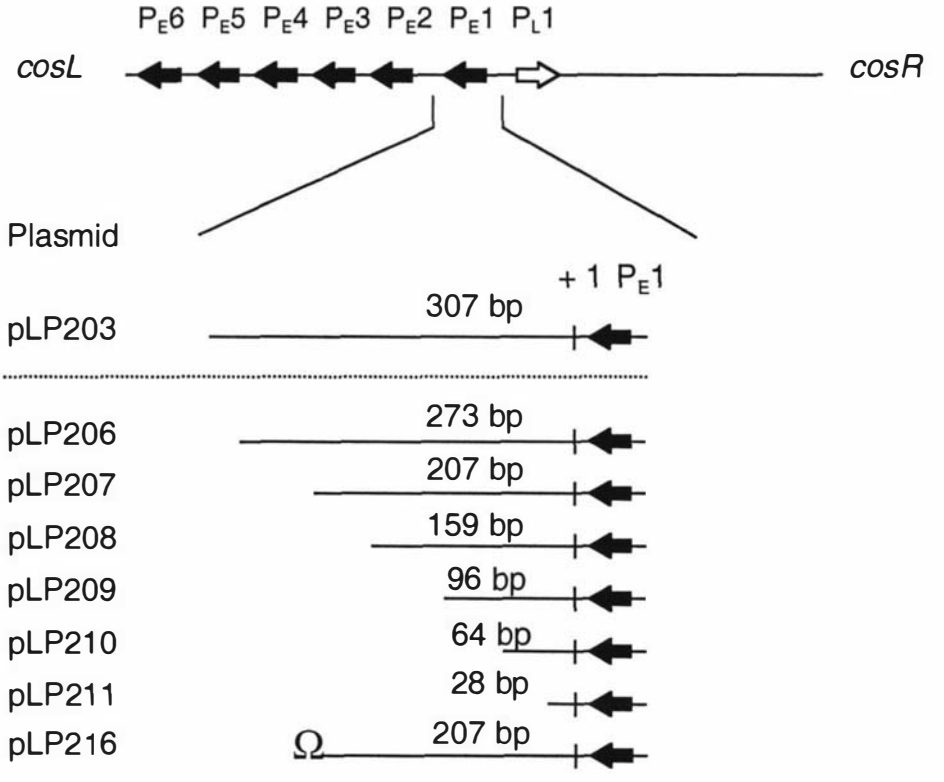


Figure 3.4. Schematic representation of the c2 *ori*-plasmids with truncated P_E1 transcripts.

The black arrow (⬅) represents P_E1 and the other early promoters, and the white arrow (⇨) represents the late promoter. Plasmid pLP203 was made by J. Gordon (Gordon, 1997). The various c2 origins with the truncated P_E1 transcripts are listed below the dotted line. In pLP216 the Ω symbol represents the transcriptional and translational terminator cloned at the 3' end of the transcript.

The pVA891 plasmids containing *ori* fragments were assayed for their ability to replicate in *L. lactis* by electroporating them into *L. lactis* MG1363 and then calculating the transformation frequency (Table 3.3). The electroporation experiment was performed three times, and water instead of DNA was used in the negative control.

The shortened transcript region in pLP206 still supported replication, but no transformants were recovered for the constructs containing a shorter DNA sequence coding for the P_E1 transcripts (pLP207-pLP211, Figure 3.4 and Table 3.4). Compared to pLP203, the transformation frequency of pLP206 was one order of magnitude lower.

Altogether, these results suggested that a certain minimal length of the transcript synthesized from P_E1 was necessary to support replication in the plasmid system.

Table 3.3. Transformation frequencies of *ori*-plasmids with truncated P_E1 transcripts.

Plasmid	**Average electroporation frequency of *L. lactis* MG1363 [cfu/μg][1] (S.D.)**
pLP206	2±0.4 x 10^3
pLP207	<2
pLP208	<2
pLP209	<2
pLP210	<2
pLP211	<2
pLP216	<2
water	<2

[1]Electroporation frequency was measured as the number of ery^R colonies per μg of DNA used in transformation. The data shown represent the average from three independent experiments. 500 ng of DNA was used for each electroporation. There was a slight variation in transformability between the batches of electrocompetent cells used in each experiment.

Previous sequence analysis has inferred the existence of a putative terminator 300 bp downstream of the P_E1 transcription start site (Lubbers *et al.*, 1998). To exclude the possibility of deleterious run-on transcription into the vector in the deletion mutants, the pLP207 *ori*-fragment was cloned into the *Pst*I and *Nco*I site of pVA891Ω (which contains a transcriptional terminator; Rakonjac *et al.*, 2003). The Ω terminator, a transcriptional and translational terminator originally from a bacteriophage T4 gene (Prentki and Krisch, 1984), is widely used and generally accepted in the literature. It is also functional in a wide range of hosts (Gram-negative bacteria, Frey and Krisch, 1985; *Streptococcus pyogenes*, Perez-Casal *et al.*, 1991).

The pLP207 fragment was amplified by PCR using PstI207term and NcoI207term primers, gel purified and then cloned into the *Pst*I and *Nco*I sites of pVA891Ω to create pLP216 (Figure 3.4). The insert of pLP216 was sequenced (primer LX-2) to ensure that no base pair changes had occurred. Three attempts were made to electroporate pLP216 into *L. lactis*, but no transformants were recovered from any of the experiments. These results suggested that the presence of the terminator did not

the experiments. These results suggested that the presence of the terminator did not restore replication, and that run-on transcription was not rendering the plasmid pLP207 genetically unstable (Table 3.4).

3.2.2 Sequence replacement of the transcript synthesized from P_E1

The region downstream of P_E1 is not conserved among prolate phages (Waterfield *et al.*, 1996; Rakonjac *et al.*, 2003). It was therefore of interest to examine not only whether a certain minimal length of the transcript but also whether a specific sequence was required for replication.

The P_E1 transcript coding region was replaced by a DNA fragment with a similar [G+C] content and length, and was derived from a lactococcal cell envelope proteinase gene (A-domain of the *prtP* gene; Christensson *et al.*, 2001). The A-domain of the PrtP protein is thought to be involved in regulation of proteinase activity and specificity (Siezen, 1999).

The DNA fragment derived from the *prtP* gene was designed not to be translated and as an extra precaution was cloned in the antisense direction. To achieve this, the proteinase DNA (primer AS5 and AS6prot, template pHP003) and the P_E1 promoter (primer AS7prot and 207Atermin, template pLP203) were amplified by PCR using overlapping primers and the PCR products gel purified. A final PCR reaction was performed using the external primers (primer AS5 and 207Atermin) and the products of the first PCRs to join the two PCR products together. The resulting product was digested with *Eco*RI and *Nco*I and then cloned into the corresponding restriction sites of pUC19. The insert was verified by DNA sequencing and then cloned into the *Eco*RI and *Nco*I sites of pVA891 to generate the new plasmid pLP214 (Figure 3.5). Sequencing of the pLP214 insert (primer LX-1) showed that no base pair changes had occurred.

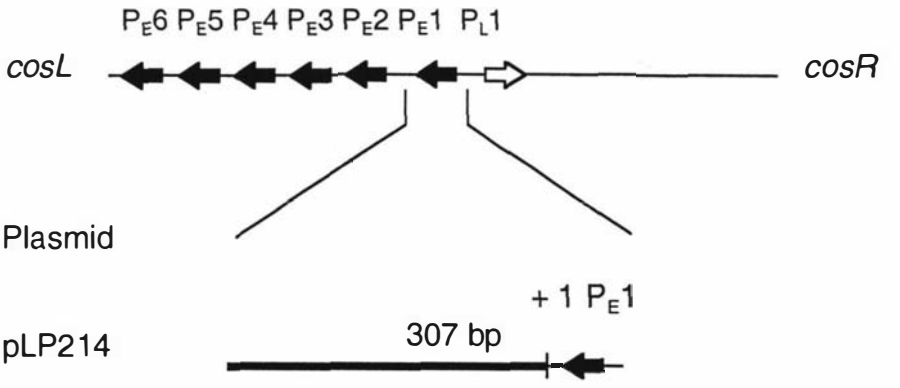


Figure 3.5. Schematic drawing of the c2 *ori* locus and pLP214.

The thick line represents the lactococcal *prtP* sequence, which replaced the c2 P_E1 transcript sequence. The black arrows (⬅) represent the early promoters, and the white arrow (⇨) represents the late promoter.

Table 3.4. Transformation frequencies of plasmids pLP214 and pLP215.

Plasmid	**Average electroporation frequency of *L. lactis* MG1363 [cfu/μg][1] (S.D.)**
pTRKH2	$3.9\pm0.3 \times 10^4$
pLP214	<2
pLP215	<2
water	<2

[1]Electroporation frequency was measured as the number of ery^R colonies per μg of DNA used in transformation. The data shown represent the average from three independent experiments. 500 ng of DNA was used for each electroporation. There was a slight variation in transformability between the batches of electrocompetent cells used in each experiment.

The plasmid pLP214 was electroporated into *L. lactis* MG1363 and assayed for its ability to replicate. Plasmid pTRKH2 was used as a positive control and water was used as a negative control in the electroporation experiments. Although the electroporation was performed three times, no transformants were recovered (Table 3.4). This result suggested that a specific sequence of the transcript is required for replication. This sequence may be critical for transcript stability or function.

3.2.3 Deletion of seven G-residues in the transcript synthesized from P_E1

The ColE1 origin of plasmid replication contains a G-tract consisting of six G-residues. It has been proposed that this G-tract pairs with a stretch of C-residues in the template strand (Masukata and Tomizawa, 1990) to promote hybrid formation of RNAII with the template DNA. The hybridized RNA is cleaved by RNase H and then used as a primer for DNA synthesis by DNA polymerase I (Itoh and Tomizawa, 1980).

A similar G-tract has also been found in the P_E1 transcript of the prolate-headed c2 phages c2, bIL67, c6A and 923. Phage c2 contains a G-tract consisting of seven G-residues, 923 has eight G-residues and bIL67 has seven G-residues interrupted by the nucleotides CTA (Rakonjac *et al.*, 2003). However, in contrast to ColE1, sequence analysis of the c2 *ori* and its flanking sequences did not reveal a complementary C-tract in close enough proximity to the G-tract to facilitate base pairing with the template DNA.

In order to examine if the G-residues play any role in c2 replication, the seven G-residues were deleted by PCR using pLP203 DNA as a template and primers latedel and JR225 (the seven G nucleotides were deleted in the JR225 primer). The PCR product was gel purified, digested with *Eco*RI and then cloned into the *Eco*RI site of pUC19. The pUC19 insert was then sequenced and recloned into the *Eco*RI site of pVA891 to create the new plasmid pLP215 (Figure 3.6) and the insert verified by DNA sequencing (primer LX-1).

Three attempts were made to electroporate the plasmid into *L. lactis,* but no transformants were recovered from any of the electroporation experiments (Table 3.4). For these experiments, the plasmid pTRKH2 was used as a positive control. The negative electroporation results obtained using pLP215 suggested that the modified c2 *ori* in pLP215 cannot support plasmid replication. A possible reason is that deletion of the seven G-residues results in a change of RNA secondary structure and therefore prevents DNA-RNA hybrid formation.

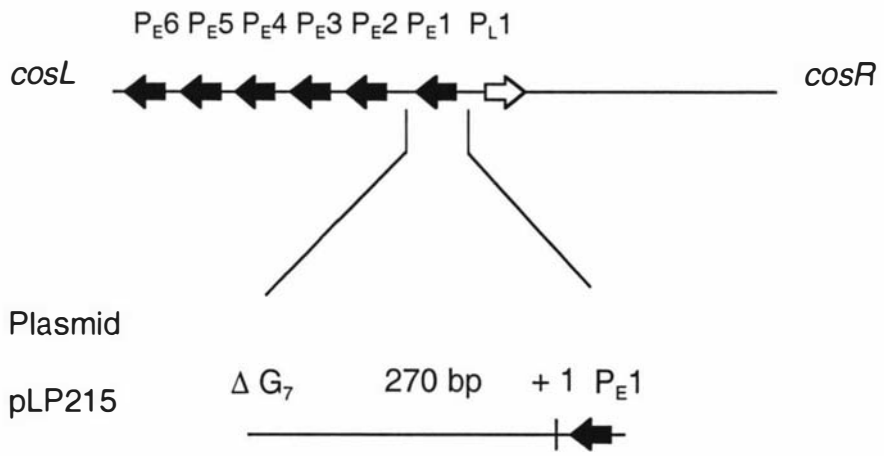


Figure 3.6. Schematic representation of the c2 *ori* locus and pLP215.

Plasmid pLP215 contains a deletion of seven G nucleotides in the P_E1 transcript (Δ G_7). The black arrows (⬟) represent the early promoters, and the white arrow (⇨) represents the late promoter.

3.3 Analysis of the P_E1 transcripts in *ori*-plasmids which support replication in *L. lactis*

3.3.1 Transcript analysis by primer extension

The transcription start site of the noncoding transcripts synthesized from P_E1 was determined in phage c2 (Lubbers *et al.*, 1998). To investigate if transcription from P_E1 in the plasmid system starts from the same nucleotide as in phage c2, primer extension analysis was performed using total cellular RNA of *L. lactis* MG1363 containing pLP201 or pLP203 (as described in section 2.10.2).

Oligonucleotide 6902prex was designed with a suitable distance from the assumed transcription start site to facilitate good gel separation of the primer extension product. The primer for the above experiment was purchased pre-purified by polyacrylamide gel electrophoresis (PAGE) to eliminate interference of primer derivatives with cDNA synthesis and detection of the transcribed product.

A single cDNA fragment was produced in the primer extension experiments using the 6902prex primer. In the negative control, RNA was omitted from the reaction and no product could be detected. The transcription start site was thus mapped to a nucleotide corresponding to the same A nucleotide identified as the transcription start in phage c2 infection (Lubbers *et al.*, 1998). The cDNA products generated are depicted in Figure 3.7, while Figure 3.8 shows the transcription start site and the P_E1 transcript sequence.

In summary the experiment showed that there is only one transcription start site downstream of P_E1 for pLP201 and pLP203 and that the cloned P_E1 is active.

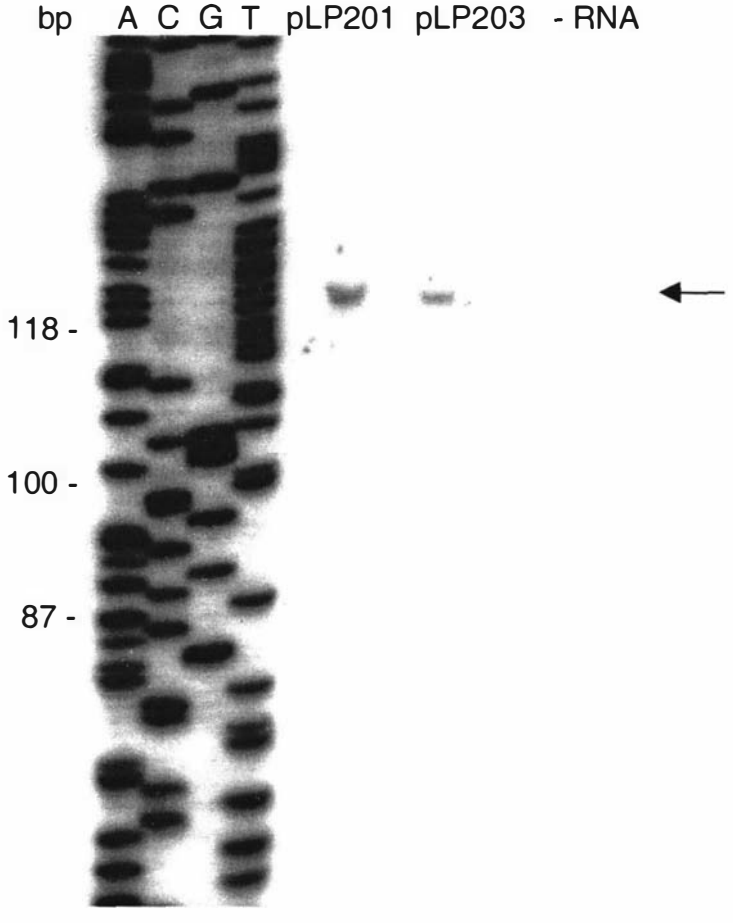


Figure 3.7. Primer extension analysis of the plasmids pLP201 and pLP203.

The primer extension reaction was performed on total RNA isolated from *L. lactis* containing plasmids pLP201 or pLP203 using primer 6902prex (lanes pLP201 and pLP203 respectively). The primer extension products can be seen in both lanes as indicated by the arrow. The same primer was used for the sequencing reaction on pLP201. Sizes in bp are indicated from the migration of end-labelled ØX174 restriction fragments (Primer Extension System, Promega).

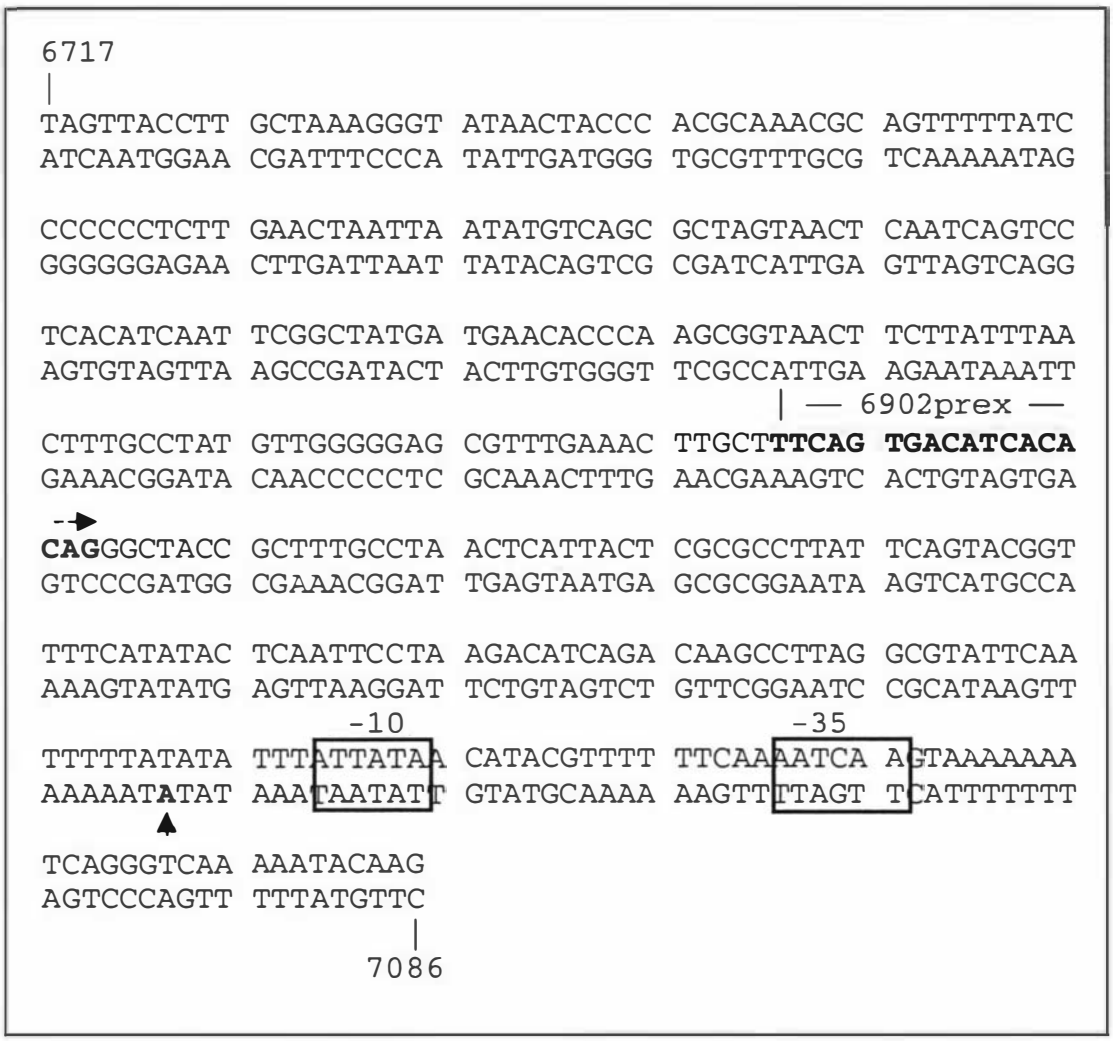


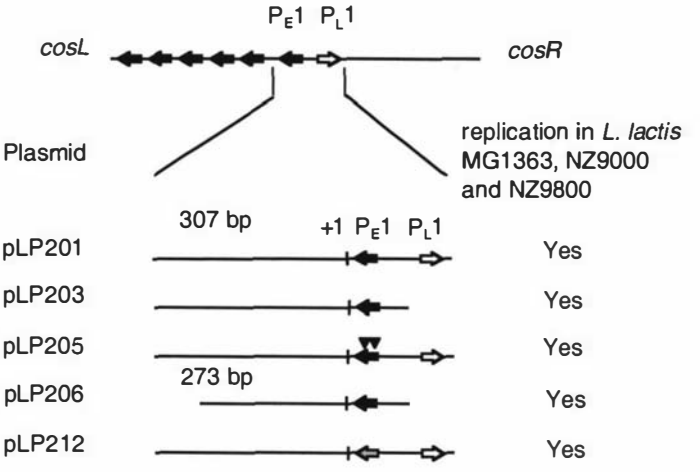
Figure 3.8. The transcription start site and the P_E1 promoter of the *ori*-plasmids, using pLP203 as an example.

The transcription start site identified by primer extension reactions is indicated by an arrow, the -10 and -35 promoter sequences of P_E1 identified by Lubbers *et al.* (1998) are boxed, and the sequence of the 6902prex oligonucleotide used for primer extension reactions is shaded. Coordinates of the *ori* sequence in phage c2 are indicated at the beginning and the end of the pLP203 *ori* insert.

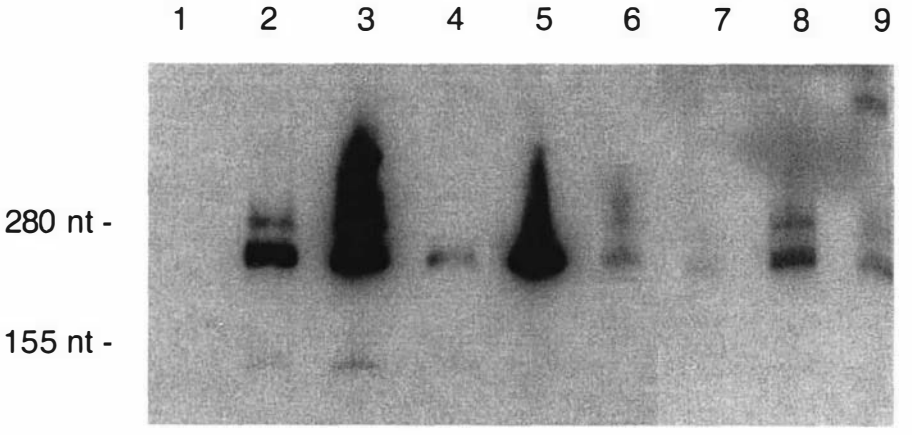
3.3.2 Northern blot detection of P_E1 transcripts synthesized from the recombinant *ori* plasmids replicating in *L. lactis*

The *ori* fragment with the mutated P_E1 promoter (pLP205) and the *ori* fragment with the *nisA* promoter (pLP212), both supported plasmid replication in *L. lactis*. However, the inability to introduce pLP204 (P_E1 deletion, Figure 3.1 and Table 3.1) into *L. lactis* strongly suggested that a functional promoter was necessary for replication. The deletion analysis of the sequence coding for the P_E1 transcript suggested that a transcript of a certain minimum length and also of a specific sequence or conformation was required for replication. Northern blot analysis was performed to confirm that the promoters in pLP205 and pLP212 were still functional and to determine the length of the transcripts synthesized from P_E1 and the *nisA* promoter.

After electroporation of the replicating *ori*-plasmids into *L. lactis*, plasmid DNA was isolated and the inserts sequenced to ensure that no base pair changes had occurred. Total cellular RNA was isolated from the *L. lactis* strains containing the replicating *ori*-plasmids (section 2.10.2), the RNA separated by gel electrophoresis and analyzed by Northern hybridization. RNA separation was attempted using two different methods. Using the first method, separation was attempted by conventional formaldehyde/agarose gel electrophoresis (section 2.10.3.1). A 2% agarose gel was used to separate the short transcripts (from 273-307 nt). However, due to the high percentage of agarose in the gel, the migration of the RNA was too slow to ensure its efficient transfer from the gel to the nylon membrane. To circumvent this problem, the RNA was separated on 6 M urea/6% polyacrylamide gels (section 2.10.3.2). The RNA was then transferred onto a positively charged nylon membrane by electroblotting (section 2.10.3.4). After crosslinking the RNA to the membrane, the RNA was visualized by staining the membrane with methylene blue to examine the quality of the RNA and RNA loading. The stained RNA ladder (0.16-1.77 kb RNA ladder, Life Technologies) was cut from the membrane and used later to determine the size of the P_E1 transcripts.



A



B

Figure 3.9. P_E1 transcript detection by Northern blot of total cellular RNA of *L. lactis* containing the replicating *ori*-plasmids.

A. Schematic representation of the pVA891-*ori*-plasmids replicating in *L. lactis*. **B.** Northern blot. Every lane contains 30 μg of total RNA. Lane 1: *L. lactis* MG1363 only; lane 2: pLP201; lane 3: pLP203; lane 4: pLP205; lane 5: pLP206; lane 6: pLP212 in NZ9800 + nisin; lane 7: pLP212 in NZ9800 - nisin; lane 8: pLP212 in NZ9000 + nisin; lane 9: pLP212 in NZ9000 - nisin. The RNA standard is indicated at the left. All RNA was isolated from *L. lactis* MG1363 unless otherwise specified.

This alternative method considerably improved both the RNA separation and the transfer of RNA onto membrane. After destaining the membrane, the P_E1 transcripts were detected by using an ECL™ labelled *ori* PCR product (primers latedel and LPS, template pLP203). Figure 3.9.A shows a schematic diagram of the plasmid templates used for the Northern blot assay. The Northern blot was repeated two times and it was demonstrated that the results from the Northern blot were reproducible. Figure 3.9.B shows a representative Northern blot and Figure 3.10 the quantitative analysis of this blot.

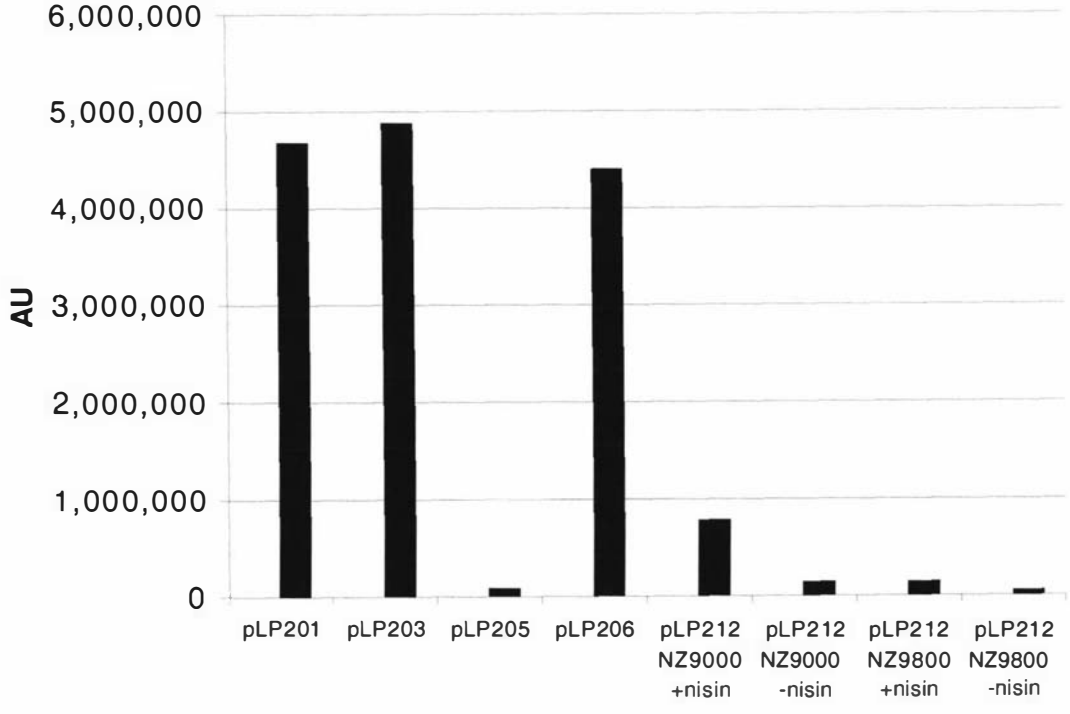


Figure 3.10. Quantitative analysis of Northern hybridization data for the P_E1 transcripts.

The light emission of the ECL™ labelled probe bound to the P_E1 transcripts (260 nt and 280 nt band) was measured with the Luminescent Image Analyzer LAS-1000 plus (AU= arbitrary units, derived from calculations on the sensitivity, latitude and contrast of the image data read).

The P_E1 transcripts could be detected in all the *ori*-plasmids (Figure 3.9.B). However, the Northern blot analysis detected more than one band for some plasmids. The major band had a length of approximately 260 nt, while a fainter band of 280 nt was detected for pLP201 and pLP203 in MG1363, and also for pLP212 in strains NZ9000 and NZ9800 in the presence of nisin.

The transcripts from pLP212 in the absence of nisin (strains NZ9000 and NZ9800) and pLP205 could be detected only after 25 min exposure of the membrane to x-ray film. No transcripts were detected in the negative control (lane 1; *L. lactis* MG1363 RNA only).

The P_E1 transcripts were quantified by a horseradish peroxidase chemiluminescent assay. The light emission from hybridized horseradish peroxidase labelled probe was measured by the Luminescent Image Analyzer LAS-1000 plus ("intelligent dark box", Fujifilm) and the data analyzed with the program Image Gauge (Ver. 3.4, Science lab 99, Fujifilm). The smear resulting from the long exposure of the membrane to the x-ray film in lanes 3 and 5 (pLP203 and pLP206, Figure 3.9.B) was not included in the analysis. The signal strength for the detected P_E1 transcripts (listed in descending order) was as follows (Figure 3.10): pLP203, pLP201, pLP206, pLP212 in NZ9000 + nisin, pLP212 in NZ9800 + nisin, pLP212 in NZ9000 - nisin, pLP205 and pLP212 in NZ9800 - nisin. The transcript signal strengths in the strains NZ9000 and NZ9800 were higher in the presence of nisin than its absence, however in these strains, even in the presence of nisin, the signal strength was considerably lower than in MG1363 containing pLP201, pLP203 or pLP206.

3.3.3 RNase protection analysis of the P_E1 transcripts synthesized from recombinant *ori*-plasmids replicating in *L. lactis*

The approximate size of the P_E1 transcripts synthesized from the *ori*-plasmids capable of replicating in *L. lactis,* were determined by Northern blot analysis. The Northern blot not only detected a major transcript of 260 nt, but also a minor, longer RNA band of approximately 280 nt. To determine the length of the P_E1 transcripts more accurately, RNase protection assays were performed. Total cellular RNA was isolated from the *L. lactis* cells containing the replicating *ori*-plasmids (section 2.10.2). Plasmid pGEMc2 (vector pGEM®-3Zf containing 419 bp of the P_E1 transcript coding sequence from +1; Rakonjac *et al.*, 2003) was linearized by restriction digest with *Bam*HI at the 3' end of the cloned P_E1 transcript. Radiolabelled antisense RNA to the *ori* transcripts was produced by *in vitro* transcription of pGEMc2 DNA with T7 polymerase (section 2.10.3.8) and [α^{32}-P] CTP. The radiolabelled antisense RNA was gel purified and then

hybridized to 10-15 μg total lactococcal RNA. The RNA:RNA hybrids were then digested with RNase ONE™, which is specific to ssRNA. The protected RNA:RNA hybrids were separated on a 5% polyacrylamide/8 M urea gel and antisense RNA was then detected by autoradiography.

The RNase protection experiment detected the P_E1 transcript in all strains carrying the replicating *ori*-plasmids, however multiple bands were present in every lane. Figure 3.9.A shows a schematic diagram of the plasmid templates used for the RNase protection assay. Three major bands of sizes 260 nt, 280 nt, and 295 nt were observed for all the recombinant *ori*-plasmids with the exception of pLP206, which produced predominantly the 260 nt transcript and a distinct slightly larger band of approximately 270 nt (Figure 3.11). The major transcripts, which all ended within the c2 insert sequence, corresponded to those detected in c2 phage infected cells (J. Rakonjac). In phage c2 an additional band of ~ 365 nt can be seen. No transcripts were detected in the negative control (lane 1, containing *L. lactis* MG1363 RNA and probe).

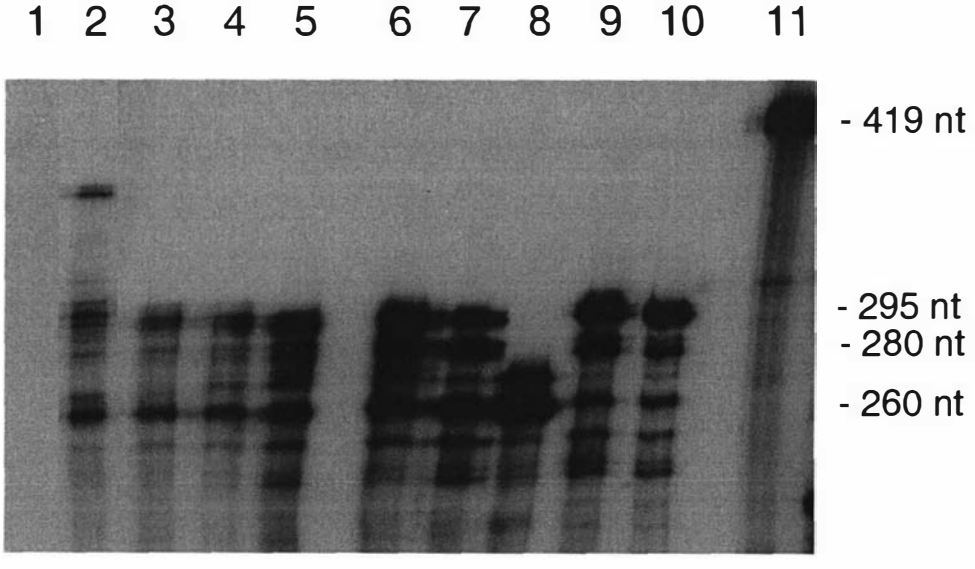


Figure 3.11. RNase protection analysis of the *ori*-plasmids replicating in *L. lactis*.

Lane 1: *L. lactis* MG1363; lane 2: phage c2 (MG1363); lane 3: pLP205 (MG1363); lane 4: pLP212 in NZ9800 - nisin; lane 5: pLP212 in NZ9000 - nisin; lane 6: pLP212 in NZ9000 + nisin; lane 7: pLP212 in NZ9800 + nisin; lane 8: pLP206 (MG1363); lane 9: pLP203 (MG1363); lane 10: pLP201 (MG1363); lane 11: radiolabelled antisense RNA of the P_E1 transcript.

3.4 Secondary structure modelling

A possible explanation for the multiple bands observed in the RNase protection experiment is that the P_E1 transcripts are processed at distinct nucleotides or particular secondary structures. Secondary structures in RNA also control the stability of mRNA, for example secondary structures at the 3' end of a transcript can enhance transcript stability as it protects the RNA from degradation by the degradosome complex (for review see Régnier and Arraiano, 2000).

Previous experiments have shown that transcription from P_E1 is necessary for replication and that the P_E1 transcript has to meet certain sequence requirements, possibly for the formation of secondary structure. Most RNAs have to fold into complex structures in order to carry out their functions. In the plasmid ColE1, RNA II forms a persistent hybrid with the template DNA and is then cleaved by RNase H to serve as a primer for DNA synthesis. In order to form this hybrid, the RNA has to fold into a complex secondary structure (Masukata and Tomizawa, 1990).

An example of RNA function being dependent on RNA tertiary structure is provided by phage Qβ, which has to fold its RNA genome into a long-range pseudoknot before it can be replicated (Klovins and van Duin, 1999). It is believed that the function of the pseudoknot is to position the 3' end of the RNA close to the phage replicase which is bound to an internally located binding site. Thus both RNA secondary and tertiary structure is important for replication.

The likely secondary structure of the P_E1 transcripts was investigated by using RNA secondary structure prediction algorithms.

3.4.1 Secondary structure modelling of the P_E1 transcripts

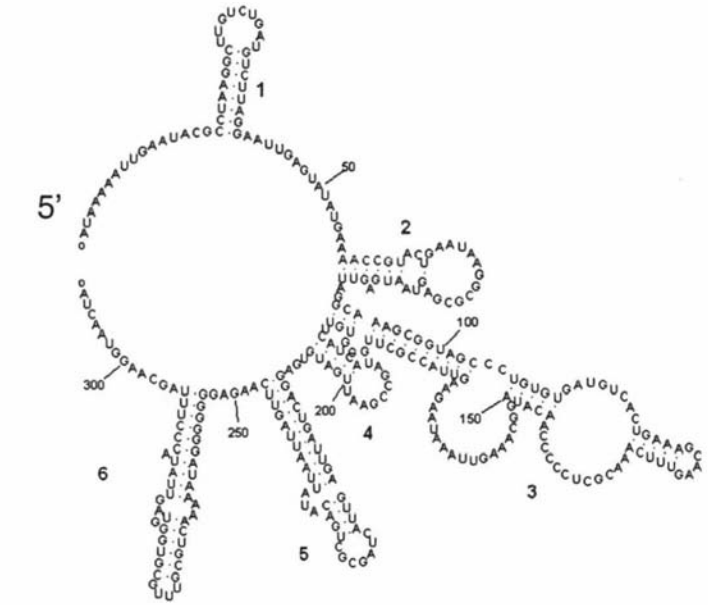
Secondary structure modelling of the 273 nt (pLP206) and 307 nt transcripts (pLP201, pLP203, pLP205 and pLP212) was aided by the data of the RNase protection experiment, which showed the length of RNA fragments potentially resulting from nuclease processing. The secondary structures were modelled using the theoretical length of the P_E1 transcripts of the various constructs with the programs mfold (Zuker

algorithm; Mathews *et al.*, 1999), RNAfold (Walter *et al.*, 1994; Mathews *et al.*, 1999), and RNAdraw (Matzura and Wennborg, 1996). The structures with a low ΔG value (i.e. the calculated, most stable secondary structures), were considered in conjunction with the data from the RNase protection assay. For overlapping structures, the output files (ct files) were imported into RnaViz (de Rijk and de Wachter, 1997) and redrawn.

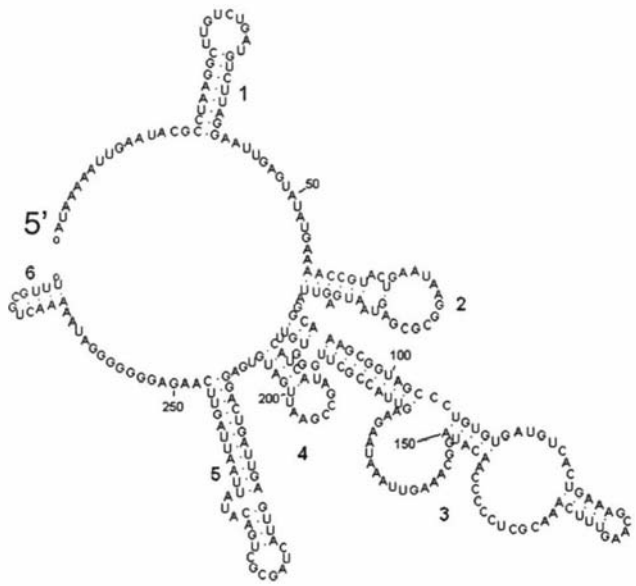
Figure 3.12 shows representative modelled structures of the 307 nt (pLP201, pLP203, pLP205 and pLP212) and 273 nt (pLP206) transcripts. Both structures can form a hairpin loop at the 5' end and differ only at the 3' end. The 307 nt transcript shows a stem-loop structure towards the 3' end, while modelling of the 273 nt transcript showed the last 11 nucleotides to form a hairpin loop.

No experimental data from RNase protection experiments were available for either the truncated, or modified transcripts. Secondary structure computer modelling of the 207 nt transcript resulted in only two structures. The structure with the lowest ΔG value is shown in Figure 3.13.A. All four structures derived for the transcript from pLP215 showed the same structure from positions 94 nt to 178 nt (a long stem-loop structure with internal loops). The 3' end showed either a short hairpin loop or was bound to the 5' end, and the 5' end showed either two hairpin loops or one very short hairpin loop. The transcript secondary structure with the lowest ΔG value is shown in Figure 3.13.B. The transcript from pLP214 showed a secondary structure which was highly divergent from the secondary structures of the 207 nt and 307 nt c2 *ori* transcripts. The secondary structures of the transcript from pLP214 all formed long stems with occasional loops which joined the 3' and 5' end of the transcript together. The structure with the lowest ΔG value is shown in Figure 3.13.C.

The ability of the transcripts to fold into tertiary structures was modelled with the program PKnots, which searches for pseudoknots in folded RNA. However, the search did not reveal any potential pseudoknots in the modelled structures.



307 nt transcript



273 nt transcript

Figure 3.12. Representative modelled structures for the 307 nt and 273 nt P_E1 transcripts.

The secondary structures for the transcripts from pLP201, pLP203, pLP205 and pLP212 (307 nt transcript) and pLP206 (273 nt transcript) were modelled using mfold, RNAfold and RNAdraw. The free energies of secondary structure formation were $\Delta G_{307\ nt\ transcript}$: -48.8 kcal/mol, and $\Delta G_{273\ nt\ transcript}$: -65.83 kcal/mol.

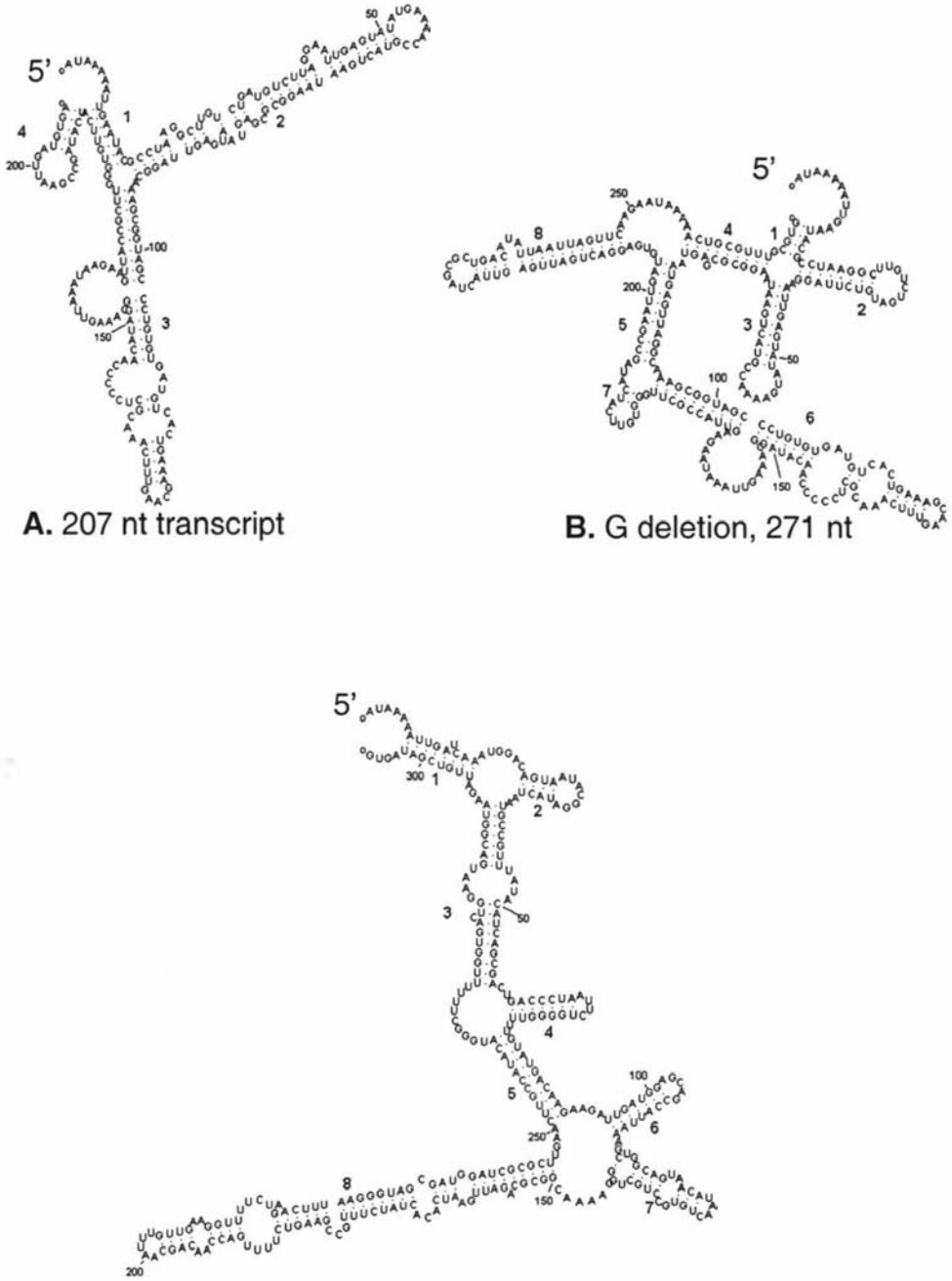


Figure 3.13. Representative modelled structures for the transcripts from pLP207, pLP215 and pLP214.

The structures were modelled using mfold, RNAfold and RNAdraw. The calculated free energies of secondary structure formation were **A.** ΔG_{pLP207}: -45.9 kcal/mol, **B.** ΔG_{pLP215}: -61.6 kcal/mol, and **C.** ΔG_{pLP214}: -77.34 kcal/mol.

3.5 Analysis of transcripts synthesized from *ori* fragments which did not support replication in *L. lactis*

3.5.1 Recloning the modified c2 *oris* into pFX3

To investigate the stability of the truncated P_E1 transcripts that did not support plasmid replication, the respective c2 *ori*-fragments of the pLP-plasmid series were recloned into the plasmid pFX3 (Xu *et al.*, 1991a), which has a functional replication origin for Gram-positive bacteria. The *ori*-fragments, which did not support pVA891 plasmid replication in *L. lactis*, as well as the c2 *ori*-inserts of pLP203 and pLP206, were recloned into pFX3 (plasmid pFX3-203 was made by J. Rakonjac). The c2 *ori*-inserts of the pVA891-*ori* plasmids were excised by digestion with the appropriate restriction endonucleases (*Eco*RI for pLP203, pLP204, pLP206, pLP207, pLP208, pLP213 and pLP215; *Eco*RI and *Sal*I for pLP214 and pLP216). After gel purification, the inserts were cloned into the appropriate restriction sites of pFX3 and the resulting constructs electroporated into *L. lactis*.

All the pFX3-*ori* plasmids could be stably maintained in *L. lactis* MG1363 except pFX3-215. The transformation efficiencies of the various constructs varied considerably (Table 3.5). The transformation frequencies for pFX3-208, pFX3-214 and pFX3-216 were approximately 100-fold lower than for pFX3-203, pFX3-204, pFX3-206, pFX3-207 and pFX3-213, indicating that there may have been a counterselection against cloned c2 *ori* inserts in these three plasmids.

Although the electroporation experiment was repeated twice, pFX3-215 could not be introduced into *L. lactis* MG1363. The plasmid pFX3 contains the pDI25 replicon (Xu *et al.*, 1991a) and replicates by a rolling-circle mechanism. It has been shown that plasmids using this mode of replication are structurally and segregationally less stable than theta replicating plasmids (Kiewiet *et al.*, 1993). For this reason, combined with the fact that some of the plasmids had low transformation efficiencies, it was important to confirm that the pFX3-*ori* plasmids had not lost the c2 *ori* inserts. Therefore, plasmid DNA was isolated from *L. lactis* containing the pFX3-*ori* plasmids and digested with the restriction enzymes used to clone them into pFX3.

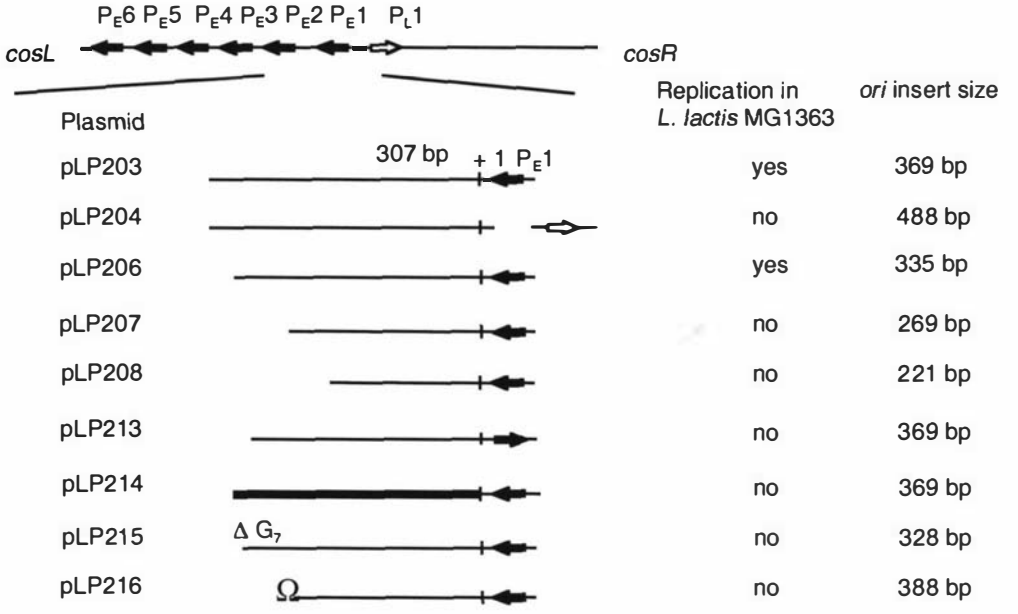
Table 3.5. Transformation frequencies of pFX3 plasmids carrying the *ori*-inserts.

Plasmid	Average electroporation frequency of *L. lactis* MG1363 [cfu/μg][1] (S.D.)
pFX3	$2.35\pm1.5 \times 10^4$
pFX3-203	$1.8\pm0.2 \times 10^4$
pFX3-204	$2.15\pm0.1 \times 10^4$
pFX3-206	$3.05\pm0.9 \times 10^4$
pFX3-207	$1.7\pm0.5 \times 10^4$
pFX3-208	$4\pm0.4 \times 10^2$
pFX3-213	$1.2\pm0.7 \times 10^4$
pFX3-214	$6.2\pm0.3 \times 10^2$
pFX3-215	<2
pFX3-216	$8\pm0.7 \times 10^2$

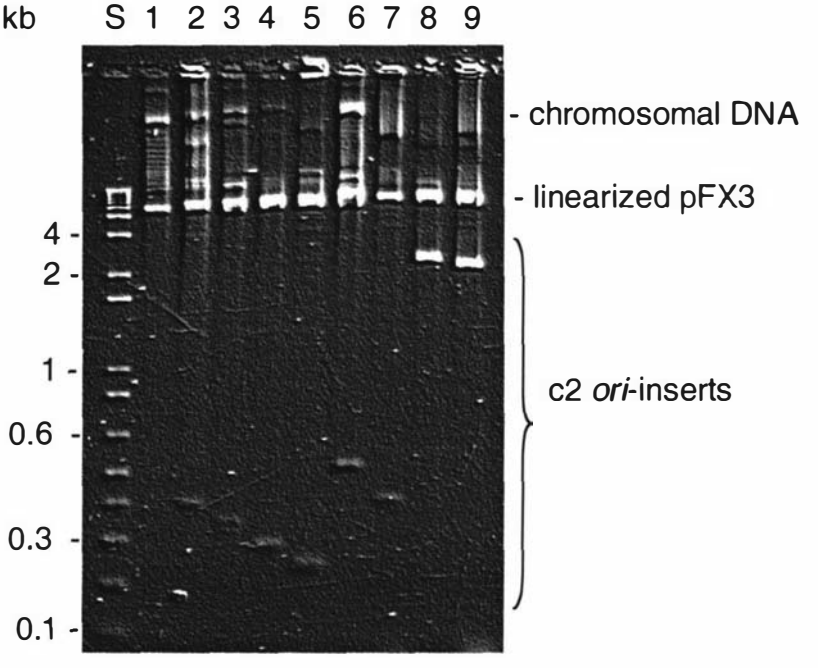
[1]Electroporation frequency was measured as the number of cm^R colonies per μg of DNA used in transformation. The data shown represent the average of three independent experiments. 500 ng of DNA was used for each electroporation. There was a slight variation in transformability between the batches of electrocompetent cells used in each experiment.

For each construct, eight transformants were checked by restriction analysis for insert size. All colonies screened for pFX3-203, pFX3-204, pFX3-206, pFX3-207 and pFX3-213 contained the inserts with the correct sizes. However, for pFX3-208, pFX3-214 and pFX3-216 only two plasmids carried the correct insert. A schematic diagram of the pVA891-*ori* constructs is shown in Figure 3.14.A, and a restriction digest of all the pFX3-*ori* plasmids with inserts of the correct size is shown in Figure 3.14.B.

All the inserts of the pFX3-*ori* clones were sequenced. None of the pFX3-*ori* plasmids that contained the P_E1 promoter in the opposite orientation to the chloramphenicol resistance gene of pFX3 (or did not contain P_E1, pFX3-204), carried mutations in the P_E1 promoter (pFX3-203, pFX3-204, pFX3-206, pFX3-207 and pFX3-213).



A



B

Figure 3.14. Restriction analysis of pFX3-*ori* plasmid DNA after isolation from *L. lactis* MG1363.

A. Schematic representation of the pVA891-*ori* plasmids and their ability to replicate in *L. lactis* MG1363. The c2 *ori* inserts were cloned into pFX3 and electroporated into *L. lactis* (Table 3.6). **B.** Restriction digest of pFX3-*ori* plasmid DNA isolated from *L. lactis* cells. Lane 1: pFX3; lane 2: pFX3-203; lane 3: pFX3-206; lane 4: pFX3-207; lane 5: pFX3-208; lane 6: pFX3-204; lane 7: pFX3-213; lane 8: pFX3-216; lane 9: pFX3-214. Lanes 1-7: *Eco*RI digest, lanes 8 and 9: *Eco*RI and *Sal*I restriction digest, adds 1.771 bp vector sequence to *ori*-insert (this figure was enhanced to show the c2 *ori*-inserts).

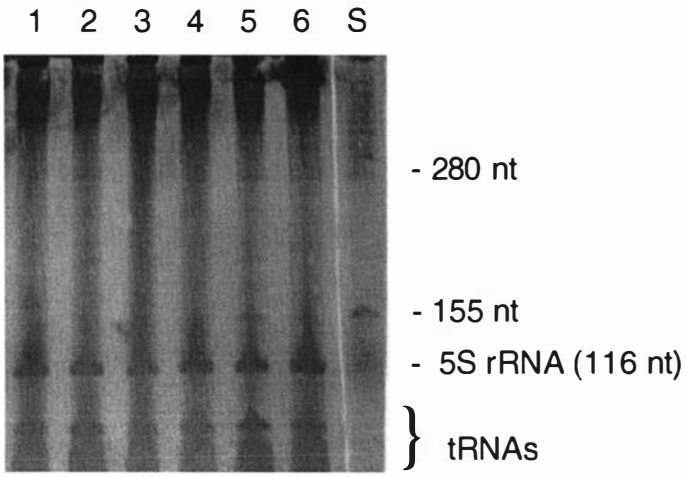
However, all the pFX3-*ori* plasmids that contained the P_E1 promoter in the same orientation as the chloramphenicol resistance gene (pFX3-208, pFX3-214 and pFX3-216), acquired mutations in the P_E1 promoter. In pFX3-208, the -10 region of P_E1 mutated from TATAAT to TGTAAT. In pFX3-214, there was a G to A mutation in the spacer region between the -10 and -35 P_E1 promoter sequence, and in pFX3-216 there was a 40 bp deletion that included the -10 region of P_E1 and the transcription start site.

3.5.2 Northern blot analysis of RNA isolated from the pFX3-*ori* plasmids

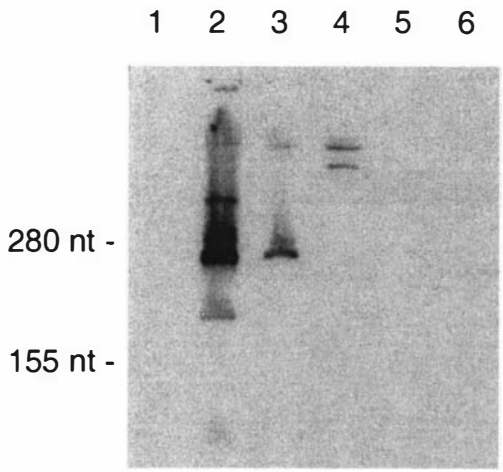
To investigate the stability of the non-functional P_E1 transcripts, total cellular RNA was isolated from all the pFX3-*ori* cultures, except pFX3-208, pFX3-214 and pFX3-216 cultures which had to be excluded as they contained mutations in the P_E1 promoter region. The RNA was separated on a 6 M urea/6% polyacrylamide gel (section 2.10.3.2) and transferred to a positively charged nylon membrane by electroblotting (section 2.10.3.4). After crosslinking the RNA to the membrane, the RNA was visualized by staining the membrane with methylene blue (section 2.10.3.4). The 5S RNA (116 nt, Nilsson and Johansen, 1994; Chiaruttini and Milet, 1993) can be seen as a distinct band on the membrane, and the tRNAs can be seen on the bottom of the membrane (approximately 69-75 nt, Figure 3.15.A); this also verified the quality of the RNA. The stained RNA ladder (0.16-1.77 kb, Life Technologies) was excised from the membrane and used later to determine the size of the P_E1 transcripts. The Northern blot was repeated two times and it was demonstrated that the results were reproducible.

The P_E1 transcripts were detected using an ECL™-labelled *ori* PCR product (primers latedel and LPS; template pLP203). The transcripts produced from P_E1 were readily detectable in the pFX3 recombinant plasmids carrying a functional c2 *ori* fragment (pFX3-203, pFX3-206; Figure 3.15.B), and showed the same pattern as the P_E1 transcripts detected by Northern blot for the pVA891-*ori* plasmids. P_E1 transcripts in the plasmids that carried a non-functional c2 *ori* fragment (pFX3-207, pFX3-213 and pFX3-204; Figure 3.15.B) could not be detected by this method. However, for pFX3-207 small amounts of P_E1 transcript that differed to that observed with the functional *ori*s (pFX3-203 and pFX3-206) were detected. This transcript was very large and was presumably the result of read-through generated from P_E1 into the plasmid. The

signal strength from detection of the P_E1 transcripts was measured with the Luminescent Image Analyzer LAS-1000 plus (Figure 3.16).



A



B

Figure 3.15. Northern blot analysis of RNA isolated from *L. lactis* MG1363 containing the pFX3-*ori* plasmids.

A. Methylene blue stained nylon membrane of total cellular RNA from *L. lactis* containing the pFX3-*ori* plasmids (30 µg RNA per lane). S: RNA standard. Lane 1: pFX3; lane 2: pFX3-203; lane 3: pFX3-206; lane 4: pFX3-207; lane 5: pFX3-204; lane 6: pFX3-213. **B.** The P_E1 transcripts were detected with an ECL™ labelled probe. See Figure 3.13.A for labelling of the lanes. The RNA standard bands are indicated on the left.

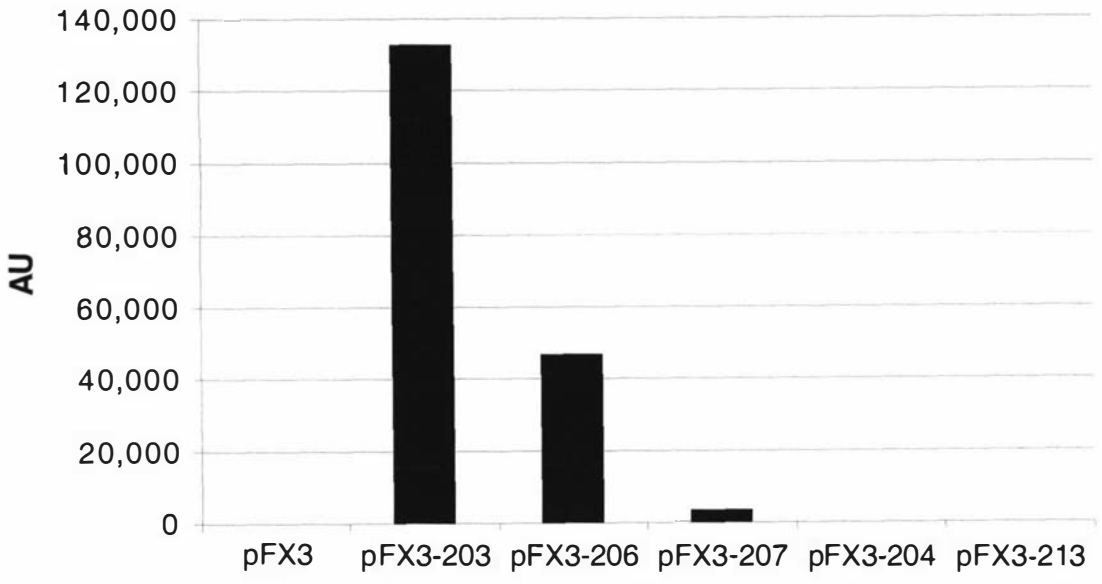


Figure 3.16. Comparison of the signal strength from the P_E1 transcript detection by Northern blot analysis of the pFX3-*ori* constructs.

The light emission of the ECL™ labelled probe was measured with the Luminescent Image Analyzer LAS-1000 plus (AU= arbitrary units, derived from calculations on the sensitivity, latitude and contrast of the image data read). The signal in pFX3-207 was derived from read-through from P_E1 into the pFX3 vector.

This analysis indicated that the steady state level was extremely low for the P_E1 transcript in pFX3-207, possibly because of rapid degradation of this transcript. This analysis also confirmed the lack of transcripts made from P_E1 in pLP204 (deletion of P_E1). No transcript could be detected in the lane with pFX3 RNA only (lane 1, negative control).

3.6 Characterization of the *ori*-plasmids replicating in *L. lactis*

3.6.1 Relative amount of *ori*-plasmid per cell

Major differences were observed in the amounts of transcript produced from P_E1 in the replicating *ori*-plasmids. Thus it was investigated whether replication was

dependent on transcriptional activity and if the amount of transcript synthesized correlated with the amount of plasmid per cell. This was achieved by determining the amount of the pVA891-*ori* plasmid per cell relative to the pTRKL2 control. *L. lactis* cultures (40 ml) containing the various pVA891-*ori* plasmids were grown to the late exponential growth phase (OD_{600} of 0.7) and the plasmid DNA isolated (section 2.6.1.3); DNA of the control plasmid pTRKL2 was isolated according to the same method. The isolated DNA was separated on a 0.8% agarose gel using 1/6 of the total amount of isolated plasmid DNA per lane. The resulting ethidium bromide stained agarose gel is shown in Figure 3.17.

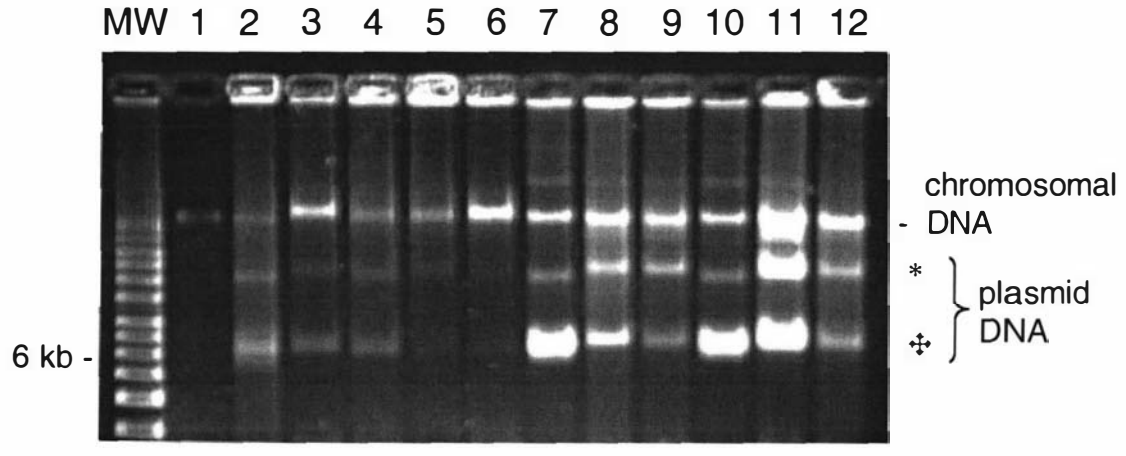


Figure 3.17. Plasmid DNA isolated from *L. lactis* MG1363, NZ9000 and NZ9800.

MW: supercoiled size marker, lane 1: MG1363, plasmid free; lane 2: pTRKL2 in MG1363; lane 3: pLP201 in MG1363; lane 4: pLP203 in MG1363; lane 5: pLP205 in MG1363; lane 6: pLP206 in MG1363; lane 7: pTRKL2 in NZ9000 + nisin; lane 8: pLP212 in NZ9000 + nisin; lane 9: pLP212 in NZ9000 - nisin; lane 10: pTRKL2 in NZ9800 + nisin; lane 11: pLP212 in NZ9800 + nisin; lane 12: pLP212 in NZ9800 - nisin; open circular * and supercoiled plasmid DNA ✣.

The relative amount of plasmid DNA was determined by measuring the intensity of the ethidium bromide stained bands by densitometry using the program NIH Image (Wayne Rasband RSB, NIH, Bethesda, MD, USA).

Although all the samples were prepared at the same time, lysis of the *L. lactis* cells did not occur uniformly, presumably because lysozyme did not function efficiently

and there were considerable differences in the amount of co-purified chromosomal DNA in the plasmid DNA preparations. It was assumed that the ratio of isolated chromosomal DNA and plasmid DNA is constant. Therefore the amount of plasmid DNA per lane (open circular form and supercoiled form in Figure 3.17) was corrected relative to the amount of chromosomal DNA per lane (all the *L. lactis* cultures were grown to an OD_{600} of 0.7). The relative amount of plasmid DNA per cell was calculated by comparing the amount of pVA891-*ori* plasmid DNA to the plasmid DNA of pTRKL2 obtained from the same lactococcal strain.

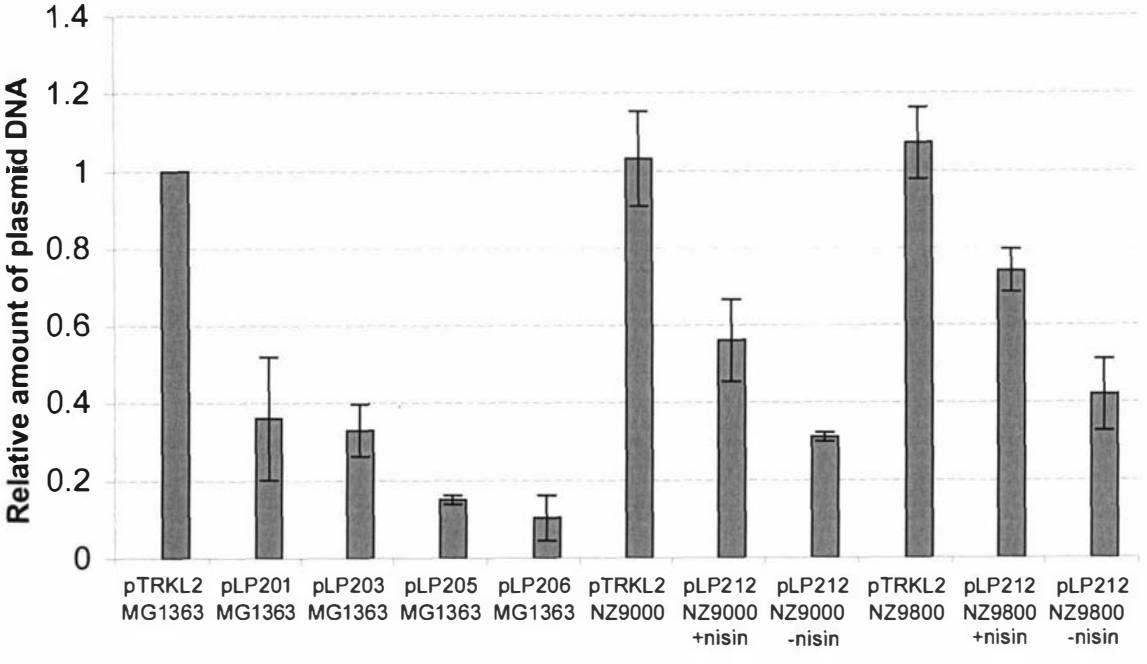


Figure 3.18. Relative amount of pVA891-*ori* plasmid DNA to pTRKL2 plasmid DNA, isolated from *L. lactis* MG1363, NZ9000 and NZ9800.

The amount of plasmid DNA (open circular form and supercoiled form) was normalized to the chromosomal DNA band. The data shown represent the average of three different experiments.

In MG1363, pLP201 and pLP203 showed the highest amount of plasmid DNA, followed by pLP205 and pLP206. The amount of pLP212 plasmid DNA in strains NZ9000 and NZ9800 was higher in the presence of nisin than without, and the relative

amount of pTRKL2 plasmid DNA isolated from MG1363, NZ9000 and NZ9800 showed only slight differences (Figure 3.18).

Because the copy number of pTRKL2 in *L. lactis* is known (6-9 copies of pTRKL2 per *L. lactis* cell; O'Sullivan and Klaenhammer, 1993) the copy number of the pVA891-*ori* plasmids could be calculated (Table 3.6). The relative amount of plasmid DNA isolated from the various *L. lactis* cells carrying the pVA891-*ori* plasmids was compared to the plasmid DNA isolated from *L. lactis* cells containing pTRKL2.

Table 3.6. Estimated copy number of the pVA891-*ori* plasmids in *L. lactis*.

Plasmid	copy number
pTRKL2	6-9 (O'Sullivan and Klaenhammer, 1993)
pLP201	2-3
pLP203	2-3
pLP205	1
pLP206	1
pTRKL2 in NZ9000	6-9
pLP212 in NZ9000 + nisin	3-5
pLP212 in NZ9000 - nisin	2-3
pTRKL2 in NZ9800	6-9
pLP212 in NZ9800 + nisin	4-6
pLP212 in NZ9800 - nisin	3-4

Unless otherwise stated, the plasmid copy number was determined in *L. lactis* MG1363.

3.6.2 Growth curves of *L. lactis* strains containing the replicating pVA891-*ori* plasmids

As discussed above, the relative amount of plasmid DNA isolated from *L. lactis* was dependent upon the pVA891-*ori* plasmids. To determine if the amount of plasmid per cell had any effect on the growth of the *L. lactis* cells with different pVA891-*ori* plasmids, *L. lactis* growth curves were determined by measuring the optical density (OD, Figures 3.19 and 3.20). The cells were diluted to a similar OD, the growth curves were performed in the presence of antibiotics (except for MG1363, NZ9000 and NZ9800 not containing any plasmids).

The relationship between cell number and turbidity is most linear between an OD of 0.05 and 0.3 thus all cells were diluted to within this range for establishing the growth curves. The growth curves for the *L. lactis* strains MG1363, NZ9000 or NZ9800 carrying various plasmids were compared. For this analysis only the time points in the exponential growth phase were considered, as the cells were diluted to similar OD values and no differences would be expected early in growth. The growth rates of the different strains were estimated as the slope of each bacterial growth curve during exponential growth (Appendix 3). To determine if differences in the growth rates were statistically significant, the ANOVA procedure of the SAS system was applied.

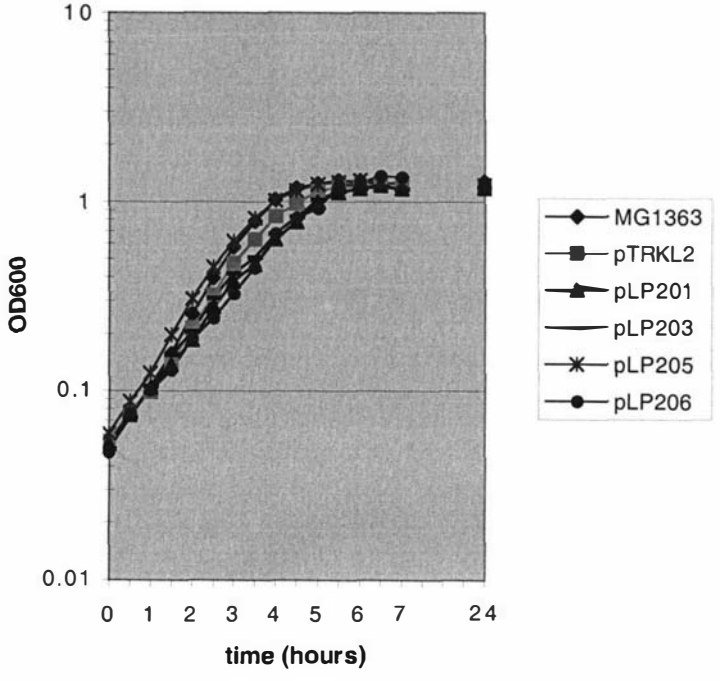


Figure 3.19. Growth curves of *L. lactis* MG1363 containing no plasmid, pTRKL2 and various pVA891-*ori* plasmids.

The growth curves represent the mean of three independent experiments, all performed from freshly electroporated cells.

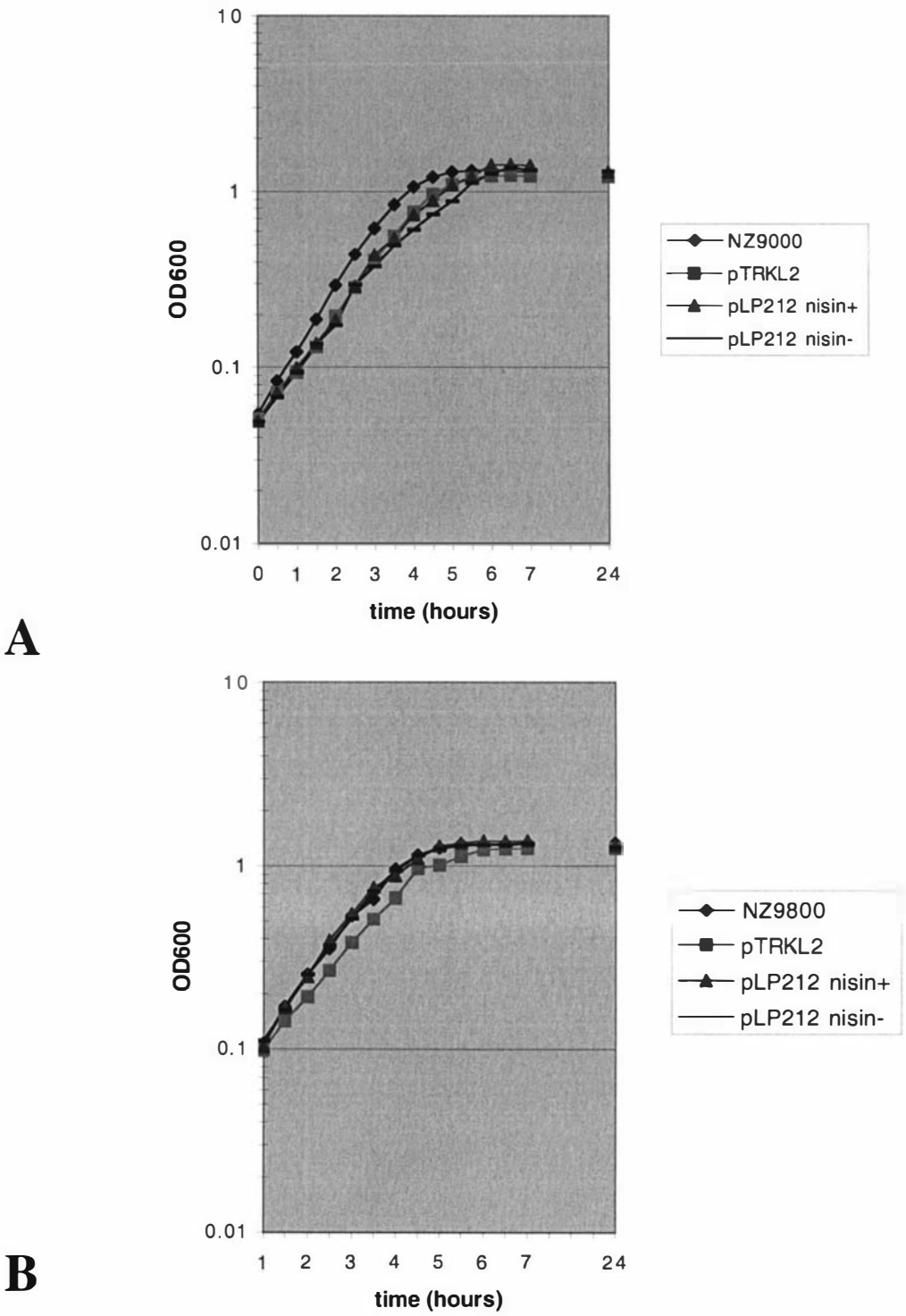


Figure 3.20. Growth curves of *L. lactis* NZ9000 and NZ9800 containing no plasmid, pTRKL2 and various pVA891-*ori* plasmids.

A. NZ9000 containing no plasmid, pTRKL2 and pLP212 in the presence and absence of nisin. **B.** NZ9800 containing no plasmid, pTRKL2 and pLP212 in the presence and absence of nisin. The growth curves represent the mean of three independent experiments, all performed from freshly electroporated cells.

A comparison of the *L. lactis* MG1363 growth rates showed that MG1363 without plasmid had a slightly higher growth rate compared to MG1363 carrying the pVA891-*ori* plasmids or pTRKL2, however this difference was not statistically significant.

The slightly different growth rates for *L. lactis* NZ9800 carrying the various plasmids or plasmid free were shown not to be statistically significant. However, NZ9800 containing plasmid pTRKL2 had a longer lag phase compared to NZ9800 containing pLP212 in the presence or absence of nisin or compared to the plasmid free NZ9800 control.

The growth rates of NZ9000 without plasmid and NZ9000 containing pTRKL2 and pLP212 in the presence of nisin were very similar. However, NZ9000 containing pTRKL2 and pLP212 in the presence of nisin had a longer lag phase than the plasmid free NZ9000 control. NZ9000 containing pLP212 in the absence of nisin had a statistically significant slower growth rate than NZ9000 containing pTRKL2, pLP212 in the presence of nisin or the plasmid free NZ9000 control.

3.7 Analysis of proteins binding to the P_E1 transcripts

The pVA891-*ori* plasmids replicate in *L. lactis* even though the cloned c2 *ori* does not encode any proteins (Waterfield *et al.*, 1996; Lubbers *et al.*, 1998). This strongly suggests that the c2 *ori* supports lactococcal plasmid replication in the absence of phage-encoded proteins. Because the sequence and length of the transcript appears to be so important for replication, it was of interest to determine if any lactococcal proteins bound to the P_E1 transcript. Binding of lactococcal proteins to the P_E1 transcript was analyzed by three methods: the electrophoretic migration shift assay (EMSA), North-Western blot analysis, and affinity purification.

3.7.1 Electrophoretic migration shift assay (EMSA)

To investigate if any lactococcal proteins bound to the transcripts synthesized from P_E1, EMSAs were performed. To prepare the sense probe, a T7 promoter was attached to the 5' end of the P_E1 transcript by PCR (Kain *et al.*, 1991) as follows. The sequence coding for the P_E1 transcript was amplified by PCR (primers 198 and LPS for the 307 probe; primers 198 and 200 for the 207 probe; template pLP203), and the T7 promoter was amplified using primers 194 and 190 (template pGEM®-3Zf(-)); primers 190 and 198 were designed to overlap. The T7 promoter and the P_E1 transcripts were joined together in a final PCR reaction using the outside primers (LPS and 194 or 200 and 194) and the PCR products of the previous PCR reactions as templates. Because *Pwo*I polymerase was used for amplification of the DNA fragments, the final PCR product could be cloned blunt ended into the *Sma*I site of pUC19 after gel purification. Sequencing of the resulting plasmids (named pT7-207 and pT7-307) confirmed that no changes occurred during amplification of the DNA sequence coding for the P_E1 transcript by PCR and also revealed the orientation of the inserts in pUC19. The plasmids were linearized with *Hinc*II and a radiolabelled transcript was produced by *in vitro* transcription with T7 polymerase and [α-^{32}P] CTP (section 2.10.3.8). Digestion with *Hinc*II added 19 bp of vector sequence to the cloned c2 sequences (207 probe: 207 bp DNA coding for the P_E1 sequence; 307 probe: 307 bp DNA sequence coding for the P_E1 sequence). The resulting lengths of the transcripts were 226 nt and 326 nt for the 207 and 307 probe respectively. The length of the transcripts was confirmed by polyacrylamide gel electrophoresis.

Cytoplasmic cell extract was isolated from *L. lactis* MG1363 (as described in section 2.11.1) and 20 µg of the extract pre-incubated with 10 µg tRNA (heterologous competitor) to block non-specific RNA-binding proteins. After adding 1-2 ng of radiolabelled RNA to the cell extract, the mixture was incubated, and the resulting RNA-protein complexes resolved by electrophoresis on a 6% nondenaturing polyacrylamide gel.

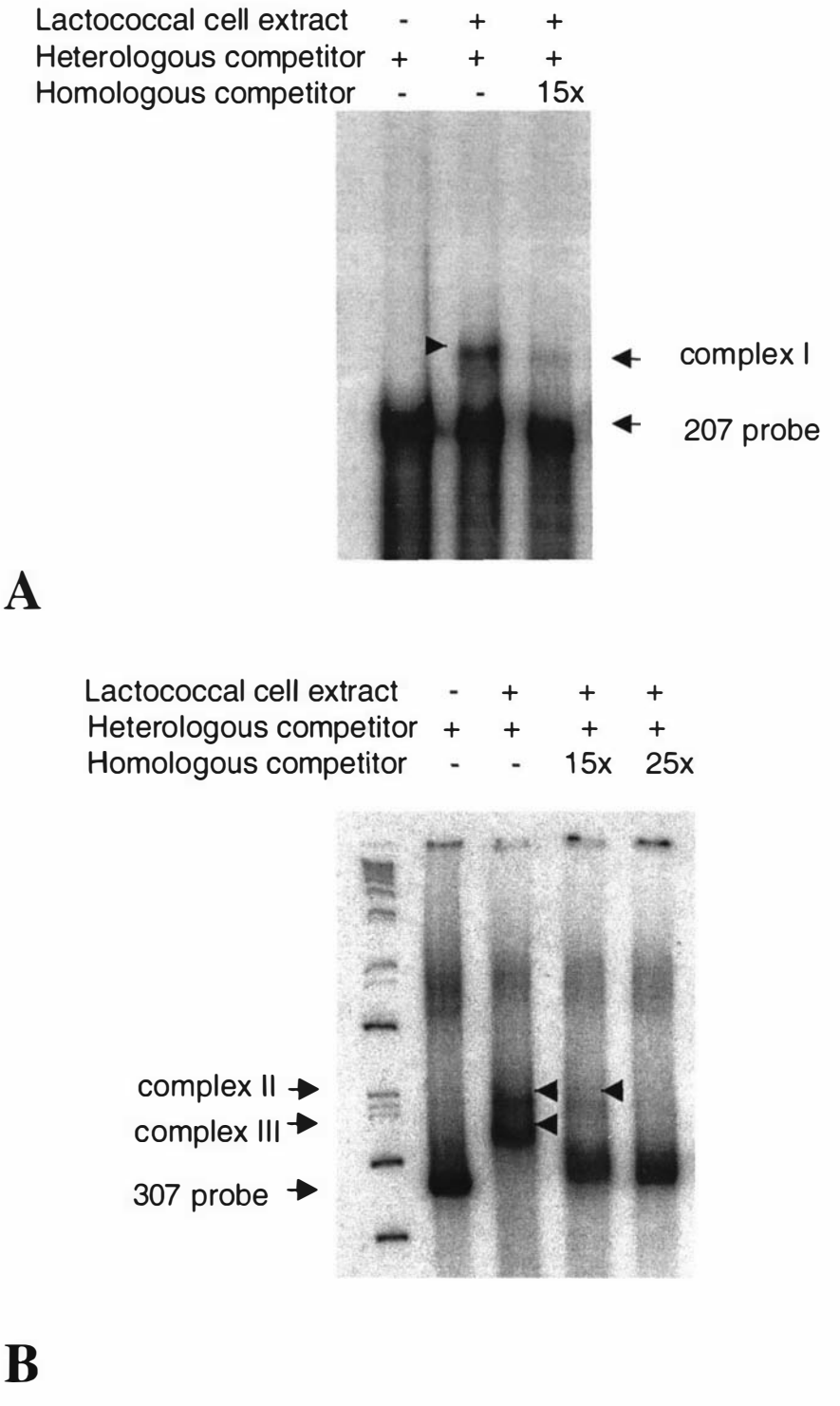


Figure 3.21. EMSAs using the transcripts synthesized from P_E1.

A. EMSA using the 207 probe. The triangle► points to a distinct band of a putative RNA-protein complex which can be seen in the presence of lactococcal cell extract and heterologous competitor (tRNA). Homologous competitor (unlabelled P_E1 transcript) competes for the complex, indicating specific RNA-protein binding. **B.** EMSA using the 307 probe. The triangles◄ point to two bands of RNA-protein complexes which can be seen in the presence of lactococcal cell extract and heterologous competitor (tRNA). Homologous competitor (unlabelled P_E1 transcript), up to 25-fold molar excess, competes efficiently for the RNA-protein complexes. The first lane shows a radioactively labelled DNA marker.

To determine whether the RNA-protein binding was specific, unlabelled competitor RNA (homologous competitor) was included in the pre-incubation step. The unlabelled competitor RNA was synthesized by *in vitro* transcription with T7 polymerase using the pT7-207 and pT7-307 plasmids in the absence of radioactive nucleotides. The electrophoretic migration shift assay detected a distinct RNA-protein complex (complex I) when the 207 probe was present. This shift was only detectable in the presence of lactococcal cell extract, and a 15-fold molar excess of unlabelled transcript competed for the complex, indicating that the transcript-protein complex was specific (Figure 3.21.A).

Two distinct RNA-protein complexes (Figure 3.19.B) were detected when using the 307 probe, indicating that at least two proteins or protein-complexes bound to the RNA (complex II and III). A 25-fold molar excess of unlabelled transcript successfully competed for the complexes indicating that the transcript-protein complexes are specific (Figure 3.21.B). Again, no shift was visible in the absence of lactococcal cell extract.

The EMSA experiment showed that lactococcal proteins bound to both the 207 and 307 probes. However, in the case of the 207 probe, not all of the probe was bound to protein(s) and only one protein-transcript complex was detected. In contrast, all of the 307 probe was bound to protein(s) and there were two protein-transcript complexes visible. Binding of the lactococcal proteins to both probes was specific, and was demonstrated by the ability of excess unlabelled homologous competitor in the pre-incubation step to compete with the RNA-protein complexes.

3.7.2 North-Western blot analysis

To determine the number of proteins binding to the transcripts and their approximate size, UV crosslinking of the lactococcal proteins to RNA was attempted. However, the UV crosslinking experiment failed (data not shown), possibly because the efficiency of cross-linking for individual proteins to RNA is highly variable and may not be suitable for some RNA-protein interactions (Piñol-Roma *et al.*, 1989).

Thus another method was used to characterize the RNA-binding proteins: North-Western blots (section 2.11.2.3). This method has been successfully used by other groups to identify RNA-binding proteins (López *et al.*, 2000; Fernández *et al.*,

1997). For the North-Western blots, lactococcal cytoplasmic cell extract (40 μg per lane) was separated using 12% SDS polyacrylamide gels, transferred by electroblotting onto nitrocellulose membrane and the membrane cut into individual lanes. The membrane-bound proteins were renatured, and 20 μg tRNA incubated with the membrane for 30 min to block any nonspecific RNA binding proteins from interacting with the probe. It is generally accepted in the literature to use tRNA to prevent nonspecific binding (Li *et al.*, 1999; Spagnolo and Hogue, 2000; Gutiérrez-Escolano *et al.*, 2000). The sense probes were made by *in vitro* transcription from plasmids pT7-207 (207 probe) and pT7-307 (307 probe) and from a PCR product (273 probe, 273 nt long; primers 194 and LP206, template pT7-307). The radiolabelled sense P_E1 transcripts (226 nt for 207 probe, 273 nt for 273 probe and 326 nt for 307 probe) and the radiolabelled control transcript (Riboprobe T7 *in vitro* transcription kit, Promega) were then added to individual membrane strips and incubated for a further hour at RT. The membrane was then washed three times and exposed to x-ray film (section 2.11.2.3).

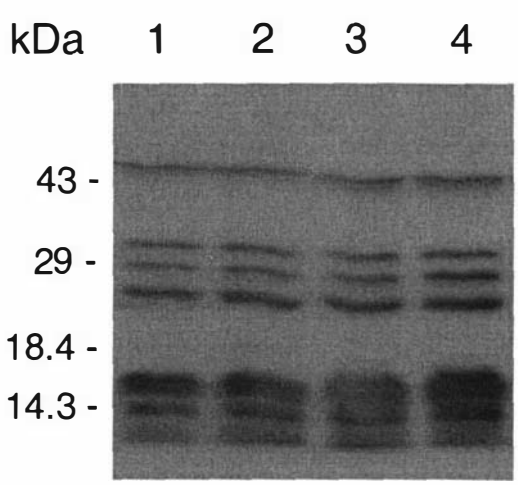


Figure 3.22. North-Western blot using the 307, 273 and 207 probes.

40 μg of lactococcal cytoplasmic cell extract was separated by SDS-PAGE and transferred onto nitrocellulose membrane. The proteins bound to the membrane were renatured and then probed. Lane 1: 207 probe, lane 2: 273 probe, lane 3: 307 probe, lane 4: control transcript.

Figure 3.22 shows that the radioactively labelled 207, 273 and 307 probes, as well as a control transcript of an unrelated sequence (T7 gene 10), all bound to the same seven proteins (estimated protein molecular weights of 13 kDa, 14 kDa, 16 kDa, 25 kDa, 29 kDa, 31 kDa and 44 kDa). Sequence analysis of the T7 gene 10 and the c2 DNA sequence coding for the transcripts made from P_E1 showed no similarity between the two sequences.

3.7.3 Purification of the proteins binding to the 307 and 207 probes

In the North-Western blot, all of the radiolabelled transcripts (including the 207, 273 and 307 probes and the control transcript) bound to the same seven proteins. The lack of specificity could have been due to the experimental protocol, particularly the blotting and renaturation steps, rather than a genuine lack of a specific RNA-protein interaction. Therefore an affinity purification protocol was attempted.

The 307 probe was biotinylated by *in vitro* transcription (plasmid pT7-307) using biotinylated UTP (section 2.11.2.4). The biotinylated RNA was then bound to streptavidin-coated magnetic particles, the particles incubated with lactococcal cytoplasmic cell extract (incubated with tRNA to prevent nonspecific binding), and any proteins bound to the RNA affinity purified by magnetic separation. Figure 3.23 shows the principle of the affinity purification.

To elute the proteins bound to the transcript, three different methods were tried: elution with high salt, digestion with RNase, and heating in SDS loading buffer. The eluted proteins were run on a 12% SDS polyacrylamide gel, and the gel silver stained to visualize the isolated proteins (section 2.11.2.1).

The proteins binding to the transcript could be effectively eluted with high salt (1 M KCl) and less effectively with RNase ONE™ digest. However, elution of the proteins using SDS loading buffer in combination with heat was not suitable for purification of the RNA binding proteins as proteins bound to the magnetic particles rather than to the RNA were released from the beads by the combination of heat and SDS (Figure 3.24, lane 4).

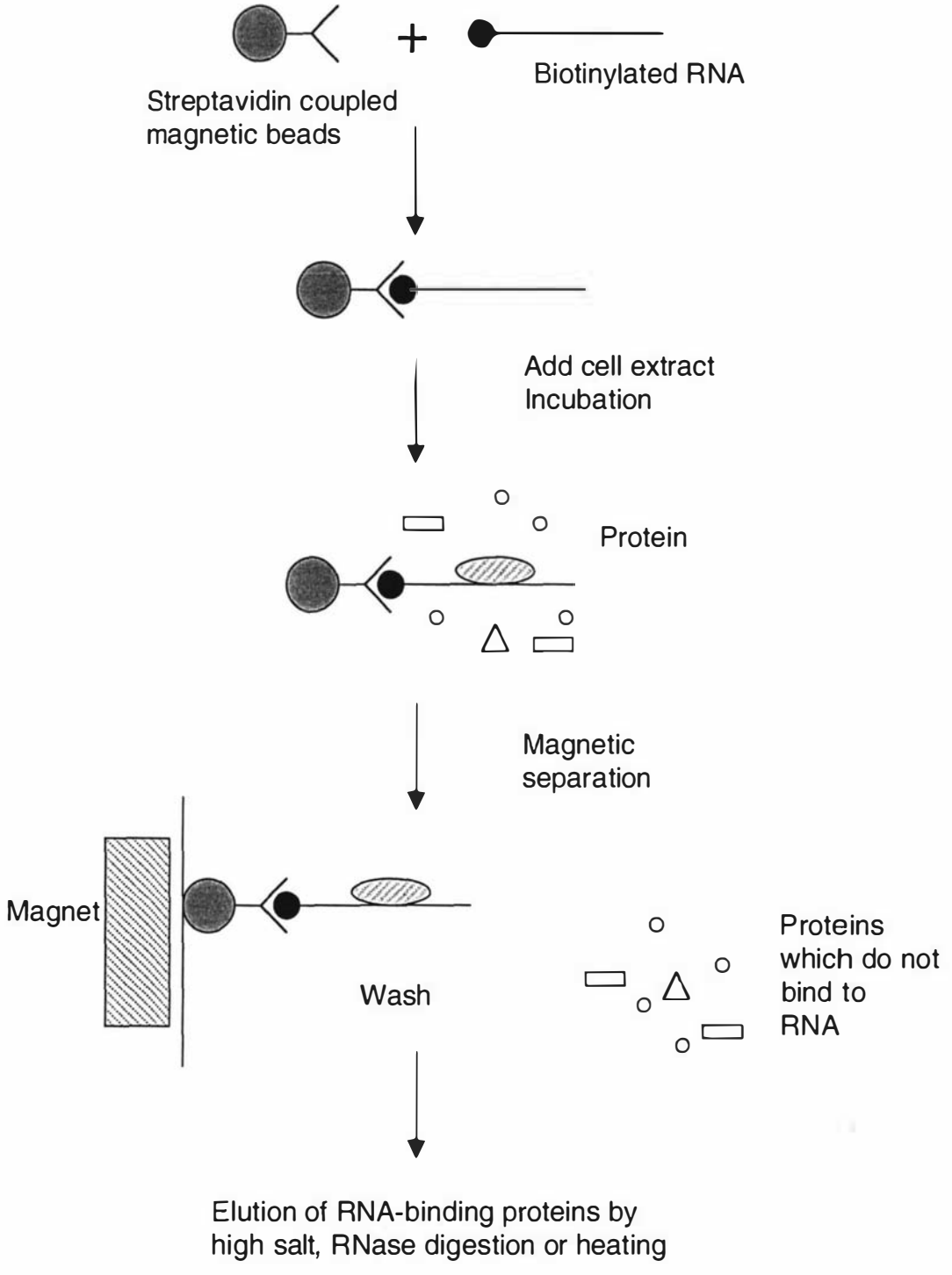
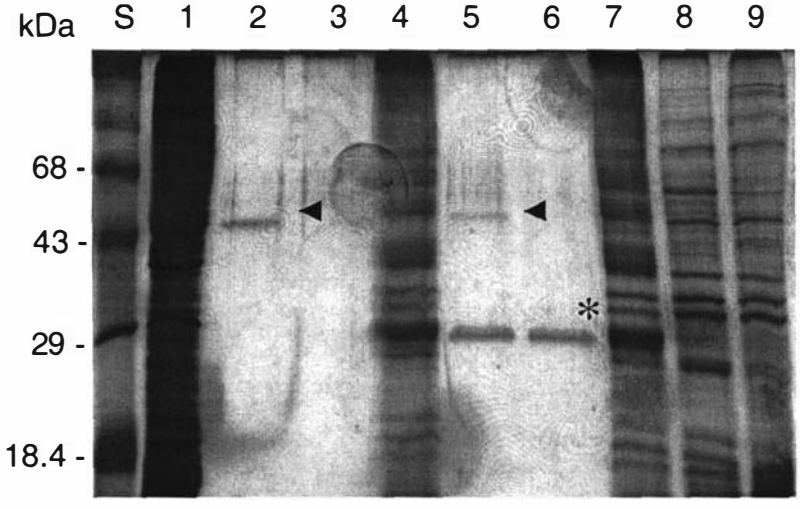
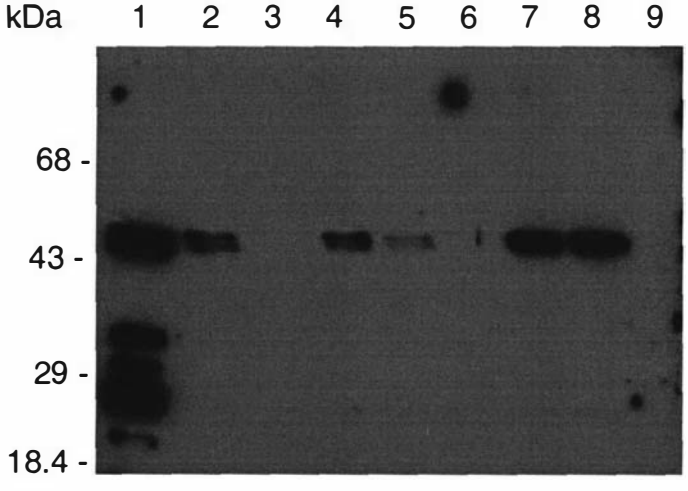


Figure 3.23. Schematic representation of the principle of magnetic RNA affinity purification of proteins.

Biotinylated RNA is coupled to streptavidin coated magnetic beads, and the beads then incubated with cell extract. RNA-binding proteins interact with the RNA and are isolated from the cell extract by magnetic separation. After washing the RNA-binding proteins are eluted.



A



B

Figure 3.24. Protein affinity purification using the 307 biotinylated probe.

A. Silver stained protein gel. Lane S: protein standard; lane 1: lactococcal cell extract (50 µg); lane 2: 307 nt transcript in the presence of lactococcal cell extract, proteins eluted by high salt (1 M KCl); lane 3: as for lane 2, without RNA; lane 4: as for lane 2, after elution by high salt, magnetic beads were boiled to elute residual proteins; lane 5: 307 nt transcript in the presence of lactococcal cell extract, proteins eluted by RNase ONE™ digestion; lane 6: as for lane 5, without RNA; lane 7: as for lane 5, magnetic beads were boiled to elute remaining proteins; lane 8: 307 nt transcript in the presence of lactococcal cell extract, proteins eluted by heating magnetic particles in SDS loading buffer; lane 9: as for lane 8, without RNA. The triangle (◀) points to the eluted 44 kDa protein binding to the 307 nt P_E1 transcript. The star * points to the 27 kDa RNaseONE™ protein. **B.** North-western blot of affinity purified proteins. The lanes correspond to Figure 3.22.A. Radiolabelled 307 nt P_E1 transcript was used as a probe.

The North-Western blot of the KCl-eluted proteins showed the same 44 kDa protein as identified by the first North-Western blot, and also a very faint band of the 25 kDa protein (lanes 7 and 8 in Figure 3.24). The negative control lanes (lactococcal cell extract in the absence of RNA) are clearly empty (Figure 3.24).

Affinity purification using the immobilized 207 and 307 probes showed the same 44 kDa protein to bind to the two transcripts (Figure 3.25).

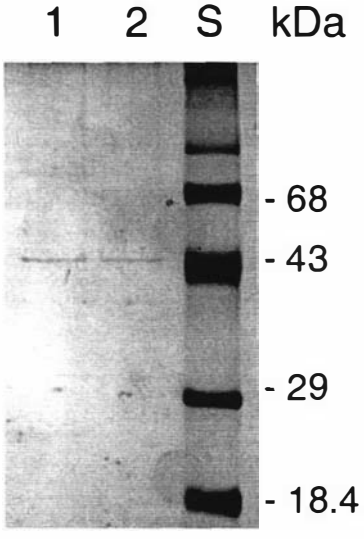


Figure 3.25. Eluted proteins after affinity purification using the 207 and 307 probes.

Affinity purification was performed using the 307 probe (lane 1) and the 207 probe (lane 2). The eluted proteins were separated on a 12% SDS gel and silver stained. Lane S contains protein standard.

To obtain sufficient protein for amino-terminal sequencing, the affinity purification was scaled up by a factor of eight. The proteins were eluted by high salt (1 M KCl), separated by SDS-PAGE (12%), transferred to PVDF protein membranes by electroblotting in CAPS buffer, and then stained with Coomassie blue. The 44 kDa and 25 kDa protein bands (labelled with a triangle and star respectively, Figure 3.26) were excised and their N-terminal sequences determined by Edman degradation.

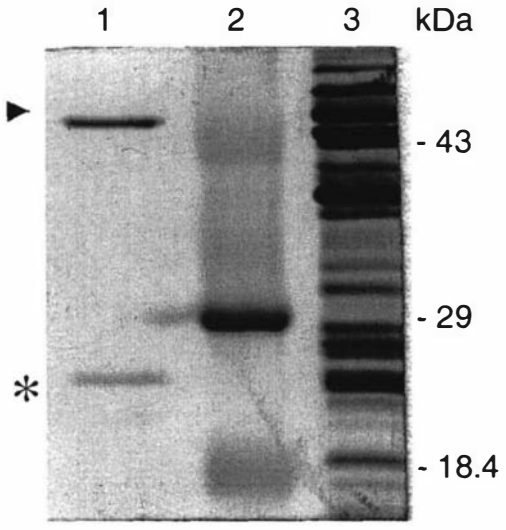


Figure 3.26. Purified proteins binding to the P_E1 transcript (preparative scale).

Lane 1: purified proteins, lane 2: protein standard, lane 3: lactococcal cell extract. The triangle ► points to the 44 kDa protein binding to the 307 nt P_E1 transcript, and the star * to the 25 kDa protein.

The amino-terminal sequence obtained for the 44 kDa protein was MNEFETLN (see Appendix 2). A database search revealed high identity with the 44.7 kDa *L. lactis* IL1403 protein S1 (MNEFETLL, Figure 4.1) (as deduced from the genome sequence of *L. lactis*; Bolotin *et al.*, 1999, 2001).

An unambiguous amino acid sequence for the 25 kDa protein which bound to both the 207 and 307 probes could not be obtained with two signals being generated for four of the first eight Edman cycles (-, R, Y/V, T/S, L/G, F/P, K, Q). Taking the size of the protein into account, the best match in a database search was a mixture of the 50S ribosomal protein L4 (22,305 Da) and the 30S ribosomal protein S4 (23,164 Da).

3.8 Investigation on whether the c2 origin confers a Per phenotype

When present at a high copy number in *trans*, the cloned origins of P335 phages ϕ50 and ϕ30 reduce efficiency of plaque formation by phages ϕ50 and ϕ30, respectively. This effect has been termed phage-encoded resistance (Per phenotype; Hill *et al.,* 1990b; O'Sullivan *et al.*, 1993). The cloned *ori* fragments are thought to interfere with phage replication by titrating out proteins essential for phage replication. The same effect has also been demonstrated for the P335 phages Tuc2009, Q30, Q33 and ul36 (McGrath *et al.*, 2001), and phage TP901-1 (Østergaard *et al.*, 2001), and for phage BK5-T (Mahanivong *et al.*, 2001). In such experiments, the plaque morphology, timing of plaque appearance, and phage titer are determined. If the plaque morphology changes, the timing of plaque appearance is delayed, or if the apparent titer decreases, this is an indicator that phage proteins or other phage factors have been titrated out by the plasmids containing the cloned *ori* fragments.

Polzin *et al.* (1999) showed that there was no Per phenotype detectable when *L. lactis* MG1363 containing pLP201 was infected with phage c2. However, the cloned c2 origin was present on a low copy number vector (pVA891, Macrina *et al.*, 1983), and therefore may have produced only a weak, non-detectable Per effect. Previous research has indicated that unless the *ori* is present on a high copy number vector, the Per effect may be too subtle to be recognized (O'Sullivan *et al.*, 1993).

To examine if the Per phenotype occurred in phage c2, the above experiment was repeated using the high copy number plasmid pFX3 (Xu *et al.*, 1990, approximately 50 copies per cell in *L. lactis*, Holzapfel and Wood, 1995). Several dilutions of phage c2 were plated on lawns of *L. lactis* MG1363 containing the various pFX3-*ori* plasmids (pFX3-203, pFX3-206 and pFX3-207) or pFX3 as a control plasmid (Figure 3.27, pFX3-207 not shown).

Neither the presence of the cloning vector pFX3 or any of the pFX3-*ori* plasmids had any effect on c2 propagation in *L. lactis* MG1363. The morphology and time of appearance of the phage plaques did not change and the phage titer did not decrease (Table 3.7).

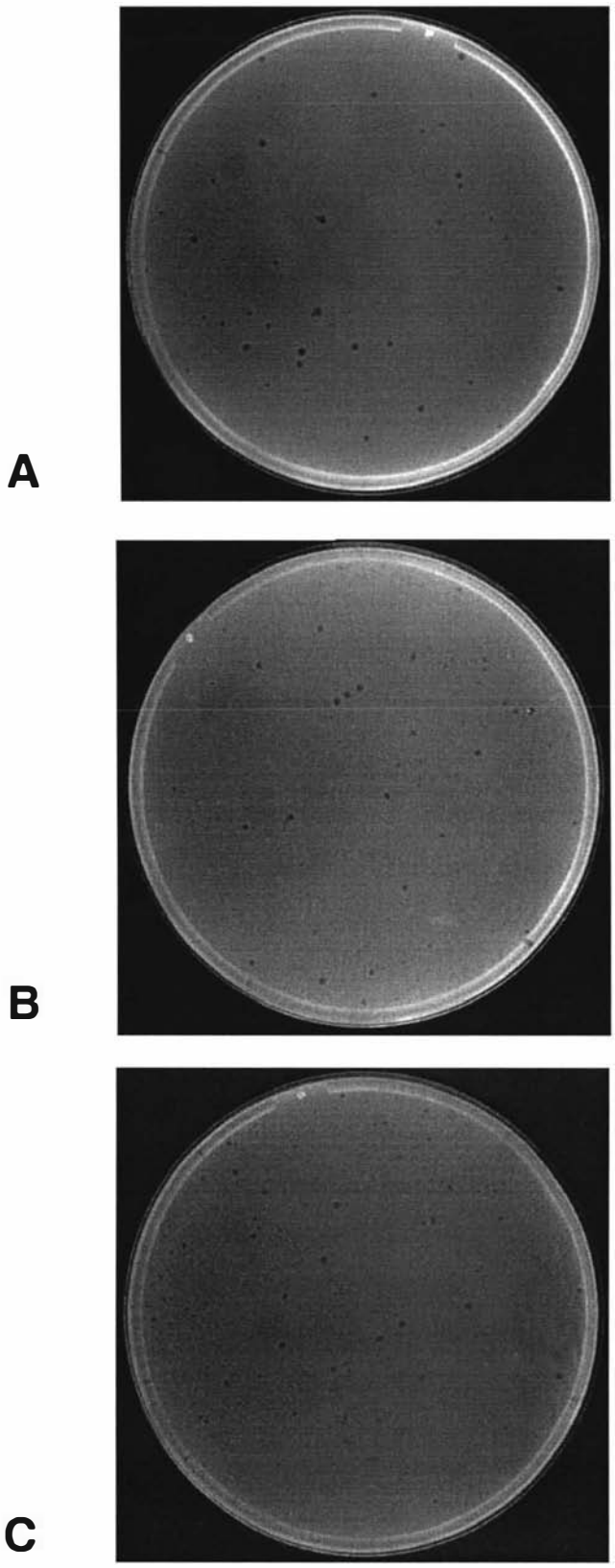


Figure 3.27. Phage c2 infection of *L. lactis* MG1363.

Plates showing plaques resulting form c2 infection of *L. lactis* MG1363 containing **A.** pFX3-203, **B.** pFX3-206 and **C.** pFX3.

Table 3.7. Phage titer of c2 infecting *L. lactis* MG1363 containing pFX3 and pFX3-*ori* plasmids.

	Phage titer of c2 infecting *L. lactis* MG1363 [pfu/ml][1]
L. lactis MG1363	$1.13\pm0.15 \times 10^{10}$
L. lactis MG1363/pFX3	$1.06\pm0.05 \times 10^{10}$
L. lactis MG1363/pFX3-203	$1.23\pm0.05 \times 10^{10}$
L. lactis MG1363/pFX3-206	$1.23\pm0.25 \times 10^{10}$
L. lactis MG1363/pFX3-207	$1.33\pm0.49 \times 10^{10}$

[1] The data shown represents the average of three independent experiments.

The effect of the various pFX3-*ori* plasmids on the efficiency of plaquing (EOP) was calculated as follows. The c2 phage titer obtained from lysis of *L. lactis* MG1363 containing the various pFX3-*ori* plasmids was divided by the titer obtained from lysis of *L. lactis* MG1363/pFX3 control. These results showed that none of the pFX3-*ori* plasmids in *L. lactis* significantly reduced the EOP of c2. This indicates that the c2 origin does not confer a Per phenotype, and nor does it titer out any phage-encoded proteins or factors. Alternatively it may be that the Per phenotype is not observed, because the proteins required for phage replication are present in abundance in the bacterial cell.

4. DISCUSSION

4.1 Major findings

In this study the requirement for transcription in lactococcal phage c2 DNA replication was investigated. The importance of the P_E1 promoter in c2 phage replication was determined by examining the effect of several P_E1 promoter modifications on replication in a plasmid system. This showed that every functional promoter that supported transcription of the sequence coding for the P_E1 transcript could support plasmid replication. The significance of the transcript synthesized from P_E1 for replication was evaluated by a series of deletions from the 3' end of the DNA sequence coding for the transcript, and by replacement of the transcript sequence by an unrelated lactococcal sequence. These experiments showed that a transcript of a certain length and sequence was required for plasmid replication in *L. lactis*.

The transcripts synthesized from P_E1 were analyzed by various methods. Primer extension experiments identified the transcription start site in the plasmid system as being identical to that in phage c2 (Lubbers *et al.*, 1998). Northern blot and RNase protection experiments detected P_E1 transcripts from all of the pVA891-*ori* plasmids replicating in *L. lactis*, although their abundance varied. RNase protection experiments also revealed that these transcripts might be processed. However, Northern blot analysis of the pFX3-*ori* plasmids carrying non-functional c2 origins failed to detect transcripts of the expected size. Modelling studies of the transcripts generated models of similar secondary structures for the functional transcripts and a diverse range of structures for the non-functional transcripts.

Host proteins were shown to bind to the P_E1 transcripts, in both electrophoretic migration shift assays (EMSAs) and North-Western blots. One of the proteins identified by North-Western blot was a 44 kDa protein which bound to the 307 nt and 207 nt transcript. Affinity purification followed by amino-terminal sequencing identified it as the ribosomal protein S1.

The plasmid DNA content of *L. lactis* varied according to which pVA891-*ori* plasmid the bacteria carried. The growth curves of *L. lactis* containing the various

pVA891-*ori* plasmids showed that the presence of these plasmids had little effect on *L. lactis* growth. Finally, it was also demonstrated that the phage c2 *ori* does not confer a Per (phage encoded resistance) phenotype.

4.2 Contribution of this study to the field

The origin of replication has been experimentally identified in several lactococcal phages: ϕ31 (O'Sullivan *et al.*, 1993), ϕ50 (Hill *et al.*, 1990b), TP901-1 (Østergaard *et al.*, 2001), Tuc2009, Q30, Q33 and u136 (McGrath *et al.*, 2001), BK5-T (Mahanivong *et al.*, 2001), sk1 (Chandry *et al.*, 1997) and c2 (Waterfield *et al.*, 1996). However, the mechanism of DNA replication and the involvement of phage and host proteins in phage replication in *L. lactis* are not well understood. This thesis presents a detailed investigation of lactococcal prolate phage DNA replication, which is characterized in c2 by a lack of requirement for phage-encoded proteins, and thus presents novel insights into this area.

The involvement of transcription in replication has been previously demonstrated for mitochondria (Côté and Ruiz-Carillo, 1993), some bacteria (*E. coli*, Baker and Kornberg, 1988), plasmids (ColE1, Itoh and Tomizawa, 1980; pAMβ1, Bruand and Ehrlich, 1998) and phages (λ, Hase *et al.*, 1989; T4, Carles-Kinch and Kreuzer, 1997). The involvement of transcription has also been suggested in phage c2 replication based on various findings: there are several transcripts synthesized from P_E1 which do not contain a ribosome binding site and are not translated (Lubbers *et al.*, 1998), and the sequence downstream of P_E1 is required for c2 replication (Waterfield *et al.*, 1996). However, much more data were required to prove this hypothesis.

The major contribution of this study is the presentation of crucial evidence for the importance of transcription in lactococcal phage c2 replication. To my knowledge, this is the first time this concept has been demonstrated in lactococcal phage replication. As shown by the P_E1 promoter and transcript analyses, a functional promoter and a minimal length and specific sequence (and possibly secondary structure) of the P_E1 transcripts were absolutely necessary for origin function.

There are two possible explanations for the involvement of transcription in replication. In the first scenario, transcription creates a transcript that can be used as a primer for replication, possibly after processing by RNA processing enzymes. Alternatively, transcription may open the DNA double helix to allow binding of a primosome that would then synthesize the actual primer (transcriptional activation). Although the involvement of transcription in c2 replication has been clearly demonstrated in this thesis, the exact role of the untranslated transcripts still remains unclear. Attempts to address this question by examining the protein binding potential of the transcripts yielded ambiguous results. The same lactococcal proteins bound to both the functional and non-functional transcripts synthesized from P_E1 and when some of the RNA-binding proteins were sequenced, all were found to be ribosomal proteins. However, there remains the possibility that it is not the transcript itself which is crucial for replication, but that transcription from P_E1 is required to open the DNA double helix and thus activates replication. This option seems less likely, given that the promoter inversion (pLP213) abolished replication and that replication only occurred in the presence of a stable transcript. However, there remains the possibility that a specific DNA sequence coding for the transcript facilitates opening of the strands or that a specific replication origin is exposed when leftward transcription occurs from P_E1. The continuing production of the transcript during c2 phage infection of the host cell (Lubbers *et al.*, 1998) suggests an on-going requirement for the transcript, possibly as a primer for leading strand synthesis, but more data will be required to substantiate this theory.

RNase protection analysis suggested that the P_E1 transcript is processed, because sequence analysis showed only one potential termination site 300 bp downstream of the transcription start site (Lubbers *et al.*, 1998). Further work will be required to determine the exact mechanism of P_E1 transcript processing.

Results presented in this thesis confirm the lack of a Per phenotype in lactococcal phage c2, as already suggested by Polzin *et al.* (1999). This result was expected, because the cloned c2 *ori* in pVA891 does not require any phage proteins for replication. This is in contrast to the several isometric-headed lactococcal phage origins belonging to the P335 and BK5-T species that have been examined to date (ϕ50,

O'Sullivan *et al.*, 1993; ϕ31, Madsen *et al.*, 2001; TP901-1, Østergaard *et al.*, 2001; BK5-T, Mahanivong *et al.*, 2001; and Tuc2009, McGrath *et al.*, 1999). The data for c2 suggest that DNA replication is entirely dependent on replication proteins of the host. This does not exclude either a role or requirement for phage proteins in the context of phage replication. As all other prolate phages examined to date (c6A, 5440, bIL67, 5447, 5449, 923 and 943) exhibit the same *ori* structure as phage c2 (Rakonjac *et al.*, 2002, submitted), it would not be surprising if there are other prolate phages that also do not show a Per phenotype. Similarly, it would also be of interest to determine if DNA replication of the small isometric-headed 936-type phage sk1 depends only on host proteins and, therefore, also lacks a Per phenotype.

4.3 Discussion of the experimental data

4.3.1 Consequences of P_E1 and transcript modifications on phage c2 DNA replication

Several replication origins of phages, eukaryotic viruses and bacteria have been examined in plasmid systems so far, including Epstein-Barr virus (*oriP*, Yates *et al.*, 2000), *E. coli* phage λ (λ*ori*, Hase *et al.*, 1989), phage T4 (ori(*usvY*), Menkens and Kreuzer, 1988), *E. coli* (*oriC*, Baker and Kornberg, 1988), and *Mycobacterium tuberculosis* (*oriC*, Qin *et al.*, 1999). For ease of manipulation, in this study the c2 origin was also investigated in a plasmid system.

In *E. coli* (Baker and Kornberg, 1988) and phage λ (Hase *et al.*, 1989) transcription from a promoter in the *ori*-region is required for DNA replication. However, current experimental data suggest that the transcripts do not act as primers, but rather that transcriptional activation is necessary for replication in these systems. In contrast, in phage T4 (Belanger and Kreuzer, 1998) and T7 (Sugimoto *et al.*, 1987) and the plasmids pAMβ1 (Bruand and Ehrlich, 1998) and ColE1 (Itoh and Tomizawa, 1980), the transcript resulting from transcription of a promoter in the origin region is thought to function as a primer for DNA replication.

Previous results (Gordon, 1997) and results obtained from this study clearly demonstrated that a functional promoter in the c2 *ori* region is required for plasmid replication. These experiments also proved that promoter activity, but not the sequence

of the promoter, was important for DNA replication, and therefore the *nisA* and P_E1 promoter leading to leftward oriented transcription could support plasmid replication. The same observation has been made in phage λ replication where the p_R promoter can be replaced by a *lac* promoter without effect on replication (Hase *et al.*, 1989).

Transcription initiation consists of several events. The σ factor/RNA polymerase complex recognizes and binds to the promoter region (close complex formation), and then the DNA strands are melted (open complex formation). The opening of the DNA strands is facilitated by topological changes in the DNA, such as negative supercoils behind the transcription forks (Liu and Wang, 1987; Mooney *et al.*, 1998; Coulombe and Burton, 1999). In the case of c2, the promoter activity may be essential for local strand melting to allow binding of replication proteins. Alternatively, promoter activity may be required for producing a transcript with a mechanistic role. This alternative is supported by the data presented in this thesis. The transcript could provide a primer for initiation of the leading strand, or could bind with DNA to form a DNA-RNA hybrid. In this scenario, the hybrid would render the non-template strand single-stranded and thus allow binding of the primosome.

Deletion analysis from the 3' end of the P_E1 transcripts revealed that there is a minimal transcript length required for DNA replication. In phage λ a certain minimal transcript length is also required for DNA replication, but the DNA sequence transcribed is not important and the transcript does not act as a primer (Hase *et al.*, 1989). This was not observed to be the case with the c2 origin. In c2, replacement of the P_E1 transcript by an unrelated lactococcal sequence abolished plasmid replication, thus confirming that the transcript is important for c2 replication.

In ColE1 replication, a tract of G-residues is important for hybrid formation of RNAII with the template DNA. It has been hypothesized that the DNA-RNA hybrid prevents rewinding of the duplex after the RNA polymerase has passed (Masukata and Tomizawa, 1990). Sequence analysis of the P_E1 transcripts of the prolate-headed phages bIL67, c6A, 923 and c2 revealed that they have a low sequence homology (13%), but they all share a conserved tract of G-residues (Rakonjac *et al.*, 2002, submitted). There are two possible explanations for why deletion of this G-tract abolished pVA891 plasmid replication in *L. lactis*. The transcript with the deleted G-tract was only 271 nt long, which is two nucleotides shorter than the shortest functional transcript (273 nt).

Thus it may be that the two nucleotides shorter length of the transcript is having a negative effect on replication. However, it seems more likely that the tract of G nucleotides is an important sequence element in the P_E1 transcript and is required for DNA replication. This sequence element may be involved in secondary structure formation of the P_E1 transcript or DNA-RNA hybrid formation as in ColE1 replication.

Several observations suggest that opening of the strands by transcription, followed by binding of replication proteins, is not sufficient for c2 replication; it seems to be more likely that transcription creates a transcript with a mechanistic role, either as a primer or through DNA-RNA hybrid formation. This is supported by the observation that only leftward transcription supported plasmid replication in *L. lactis* while transcription in the opposite direction abolished replication, and also by the fact that replication required a transcript with a minimal length and specific sequence. However, this still leaves the possibility that a specific DNA sequence coding for the transcript facilitates opening of the strands and that as in phage λ replication, opening of the strands has to proceed over a certain distance (Hase *et al.*, 1989).

Northern blot analysis of pLP205 and pLP212 showed that both promoters in the cloned c2 *ori* fragments were functional, despite the mutation of the P_E1 promoter in pLP205 from TATAAT to TAGAAG. The P_E1 transcript in pLP205 was only detected after 25-30 min exposure to the x-ray film compared to 2-5 min exposure needed for pLP201, pLP203 and pLP206. The low amount of transcript detected in pLP205 was expected because of the mutations in its P_E1 promoter, and indicated that the mutations seriously weakened the promoter activity but did not abolish activity completely. This is in agreement with the findings of Jensen and Hammer (1998) who created synthetic lactococcal promoters, cloned them upstream of the promoterless *lacL* and *lacM* genes (which code for β-galactosidase) and investigated the promoter strength by measuring β-galactosidase activity. This study also showed that single base pair changes in the -10 region of the promoter significantly reduced the strength of the promoter without completely abolishing its activity. Based on this study it can be concluded that even a weak promoter in the c2 *ori* can facilitate plasmid replication in *L. lactis*.

The *nisA* promoter was originally reported as being tightly repressed in the absence of nisin inducer (Kuipers *et al.*, 1995). The *nisA* promoter in pLP212 was active in the absence of nisin (Northern blot analysis), and the pLP212 plasmids in the lactococcal strains NZ9000 and NZ9800 replicated without nisin induction. This was unexpected because the *nisA* promoter should have been repressed to background level (Kuipers *et al.*, 1995). However, there are several examples in the literature of *nisA* promoter activity without nisin induction. Chandrapati and O'Sullivan (1999) observed that as long as lactose or galactose are present in the media, the *nisA* promoter in *L. lactis* can be induced in the absence of nisin. Furthermore Eichenbaum *et al.* (1998) showed that there was still β-glucuronidase (GUS) activity in the absence of nisin in some bacterial cells (*S. agalactiae*, *S. pneumoniae* and *B. subtilis*) containing plasmids with a promoterless *gusA* gene transcriptionally fused to the *nisA* promoter. Another example is provided by Bryan *et al.* (2000) who demonstrated residual activity of the *nisA* promoter in the absence of nisin in *Enterococcus faecalis*.

In plasmid pNZ8037, the *nisA* promoter consists of nucleotides -156 to +156 relative to the transcription start site and, unlike the pLP212 plasmid, was reported to lack activity in the absence of nisin (de Ruyter *et al.*, 1996b). The *nisA* promoter in pLP212 consists of the nucleotides -143 to -3 upstream of the transcription start site; the transcription start site and the ribosome binding site of the *nisA* gene were not included because this would have altered the P_E1 transcript. However, the *nisA* promoter in pLP212 still contained the two pentanucleotide repeats (TCTGA) between positions -39 to -24 relative to the transcription start site, which are believed to play a role in the tight control of *nisA* promoter activity (de Ruyter *et al.*, 1996a). This is in agreement with Kuipers *et al.* (1998) who maintain that a *nisA* promoter fragment comprising 40 bp upstream of the transcription start site is sufficient for nisin controlled expression. However, Christensson *et al.* (2002) also demonstrated that the *nisA* promoter was active in *L. lactis* in the absence of nisin even when the ribosome binding site and the transcription start site of the *nisA* gene was included in the *nisA* promoter construct. Therefore, it is possible that the *nisA* promoter is active in the absence of nisin at least under the conditions used in this study.

The P_E1 transcripts from all of the pVA891-*ori* plasmids replicating in *L. lactis* could be detected by Northern blot analysis, although their signal strength varied considerably. For both the NZ9000 and NZ9800 strains, the amount of P_E1 transcript detected from pLP212 was higher in the presence of nisin compared to when no nisin was present, as expected. In addition, the quantity of P_E1 transcript detected in strains NZ9000 and NZ9800 carrying pLP212 was lower than that for plasmids pLP201, pLP203 and pLP206. It is possible that P_E1 is a much stronger promoter than the *nisA* promoter. In addition, the induction efficiency of the *nisA* promoter is known to depend on the growth phase of the bacteria, and also on the amount of inducer used.

The amount of transcript detected in NZ9000 was higher than in NZ9800, indicating that the NZ9000 strain had a higher induction efficiency, which is consistent with the literature (Kuipers *et al.*, 1998). This is probably due to a proportion of the nisin supplement binding to NisI or other immunity proteins in the NZ9800 strain (Kuipers *et al.*, 1993). NZ9000 has the *nisR* and *nisK* genes integrated into *pepN* but lacks the immunity genes (Kuipers *et al.*, 1998). For this reason it was important not to exceed the MIC (minimum inhibitory concentration) value for nisaplin against NZ9000 (MIC for nisin A against NZ9000: 10 ng ml^{-1}, Kuipers *et al.*, 1998). The nisaplin used contained 10^6 international units nisin g^{-1} (or 25 mg nisin g^{-1}; Delves-Broughton, 1998).

4.3.2 Transcript analysis by primer extension, Northern blot analysis, RNase protection and secondary structure modelling

The oligonucleotide used in the primer extension experiment generated a cDNA of a single size, indicating that there was only one transcription start site in the tested pVA891-*ori* plasmids (pLP201 and pLP203). This site mapped to an A-residue in the *ori*-plasmids and was identical to the transcription start site in phage c2 (Lubbers *et al.*, 1998).

All of the P_E1 transcripts detected by Northern blot from the pVA891-*ori* plasmids, with the exception of pLP206, showed not only a major band of 260 nt, but also a minor band at approximately 280 nt. Further bands (representing the P_E1 transcripts) were detected by RNase protection experiments, presumably because of the better resolution of the RNA in a larger gel. Similarly, multiple bands were also

detected by RNase protection using phage c2 RNA, indicating that these bands were not an artifact specific to the pVA891-*ori* plasmids. Although DNA sequence analysis suggested a putative terminator 300 bp downstream of the transcription start site (Lubbers *et al.*, 1998), this fails to explain the 260 nt and 280 nt bands. Furthermore, the extra bands could not have been the result of RNA degradation by ribonuclease contamination during the RNase protection experiment as this would have resulted in a smear as opposed to the discrete bands that were observed. The control in the RNase protection analysis (Figure 3.11, lane 1, *L. lactis* MG1363 without plasmid) showed no bands, indicating that the probe was completely digested by RNase ONE™ and did not form complex structures itself. Instead the defined nature of the bands suggests either that there are more termination sites in the DNA sequence coding for the transcript than were suggested by sequence analysis, or, alternatively, that the transcript is processed at distinct nucleotides and/or secondary structures by RNA processing enzymes. All cellular RNAs are processed after transcription from DNA (Symmons *et al.*, 2002). One example of the importance of RNA processing is demonstrated in ColE1 and mtDNA replication where the transcripts have to be cleaved by ribonucleases (RNase H and RNase MRP respectively) before they can serve as primers for DNA replication (Itoh and Tomizawa, 1980; Lee and Clayton, 1998).

The relative amounts of P_E1 transcript detected by Northern blot analysis and the RNase protection experiment varied between the two methods. Some of the variation could be due to the more complicated sample preparation required for the RNase protection experiment (phenol extraction and precipitation steps).

RNA molecules not only carry the genetic information coding for proteins, but also catalyze chemical reactions (ribozymes) and recognize substrate molecules (aptamers). These functions are dependent on the three-dimensional structure of the RNA molecule. Transcript secondary structure modelling, using different computer programs, predicted that the transcripts could fold into a range of structures. Only the c2 sequences were used for modelling, no downstream vector sequences were included because RNase protection analysis and Northern blots showed that the longest detected transcript was 295 nt. The structures modelled for the 307 nt and 273 nt transcript with the lowest free energy values (ΔG, indicating stable structures) generated a hairpin structure at the 5' end for both transcripts. However, the models for the two transcripts

differed at the 3' end, with the 273 nt transcript having a hairpin structure at the extreme 3' end, and the 307 nt transcript having a stem-loop structure 11 nt from the 3' end. In the RNase protection experiment, the 307 nt transcript showed three major bands at 295 nt, 280 nt and 260 nt, which is consistent with the modelled data. The modelled secondary structure of the 307 nt transcript generated a stem-loop at position 295 that could potentially protect the RNA from 3'-5' degradation by exonucleases like PNPase and RNase II (3'-5' exonucleases, Carpousis *et al.*, 1999). According to the model, positions 260 and 280 are located in an internal loop inside a stem-loop structure, which would leave them accessible to endonucleases like RNase III (RNase III cleaves dsRNA at stem-loop structures containing few base pair mismatches; Régnier and Arraiano, 2000). For the 273 nt transcript, the RNase protection experiment showed major bands at approximately 270 nt and 260 nt. The 260 nt band could be a result of cleavage by an ssRNA processing enzyme, like RNaseE, which cuts upstream from a hairpin or stem-loop structure (Belasco and Higgins, 1988), while the 270 nt band probably consisted of unprocessed RNA. This is consistent with the modelled data, as the hairpin loop at the 3' end of the transcript could protect it from PNPase and RNase II degradation.

Modelling studies showed both transcripts to have the potential to form a hairpin at the 5' end. Confidence in the model is increased by the observation that 5' terminal secondary structure elements can increase mRNA stability (for review see Régnier and Arraiano, 2000). An example of this has been demonstrated by Carrier and Keasling (1997) who showed that the addition of a synthetic hairpin structure at the 5' end of *lacZ* mRNA increased the mRNA half-life from 2.6 min to 8.2 min. Although 5' terminal secondary structure elements are known to increase mRNA stability, to date no 5'-3' exoribonuclease has been found in bacteria (Symmons *et al.*, 2002). In addition to the RNase protection experiment, the primer extension data provided further evidence that the 5' ends of the P_E1 transcripts were not processed.

Secondary structure modelling of the non-functional 207 nt transcript (pLP207, Figure 3.27) did not reveal the 5' hairpin loop, although it did generate a hairpin loop at the 3' end. Although the 207 nt transcript and the 273 nt transcript have an identical 5' end, the secondary structures are very different because of the different length of the transcripts and the computer program calculates the most stable secondary structures. Modelling of the non-functional transcript with the deletion of seven G nucleotides near

its 3' end (pLP215) generated the same long stem-loop structure from position 96 to 176 as did the 207 nt transcript but the structures differed at both the 3' and 5' ends and also differed from the models of the functional transcripts. For the modelled secondary structure of the sequence derived from the proteinase gene (pLP214), no convincing similarities to the secondary structures of the other transcripts were revealed. The differences in secondary structure for these non-functional transcripts compared to the 273 nt and 307 nt transcripts may explain why they did not support plasmid replication. After processing, it is possible that the processed derivatives form different secondary structures and could serve as primers for DNA replication.

To date, only a few genes involved in RNA processing in *L. lactis* have been identified (Bolotin *et al.*, 2001). Some of the genes (and the proteins they encode) are: *rnhA* and *rnhB* (both encode a 3'-5' exonuclease that cleaves RNA in RNA-DNA hybrids, RNase HII homologues), *rnc* (ribonuclease III, cleaves dsRNA, Drider *et al.*, 2002), *pnpA* (polynucleotide phosphorylase, PNPase, 3'-5' exonuclease, cuts ssRNA; Duwat *et al.*, 1999), and *vacB1* and *vacB2* (members of the ribonuclease II family, 3'-5' exonuclease, cuts ssRNA). To date, no ribonuclease E has been identified in *L. lactis*, although studies of mRNA decay in *L. lactis* and *E. coli* do suggest that *L. lactis* contains an RNase E-like ribonuclease (Drider *et al.*, 1998).

A disadvantage of modelling secondary structures is that in nature the structures with the lowest free energy are not always favoured and only approximately 46% of the modelled structures for longer RNA molecules (>100 nt) are predicted correctly (for review see Higgs, 2000). Although the transcripts used to model the secondary structures were all large (in the range of 207 nt to 307 nt), the modelling of the two functional transcripts was aided by limited nuclease digestion data. Despite the fact that all models would benefit from more refinement, they do provide a feasible model for future studies.

To characterize the non-functional transcripts, the c2 *ori* fragments from the pVA891-*ori* plasmids, which could not replicate in *L. lactis*, were recloned into pFX3. However, a difficulty was encountered in the recloning step. The pFX3-*ori* plasmids that contained the P_E1 promoter in the opposite orientation to the chloramphenicol resistance gene could be transformed into *L. lactis* with a electroporation efficiency of

10^4 cfu/μg. However, pFX3-*ori* plasmids containing the P_E1 promoter in the same orientation as the resistance gene could be only transformed with a low electroporation efficiency (10^2 cfu/μg, pFX3-208, pFX3-214, and pFX3-216), or not at all (pLP215). Although restriction analysis of these pFX3-*ori* transformants indicated that they carried the inserts of the correct size, DNA sequencing showed them all to have a deletion or mutation in P_E1. For this reason pFX3-208, pFX3-214, and pFX3-216 were not included in the Northern blot analysis. However, pFX3-207 could be used for the Northern blot analysis as sequence analysis of the plasmid showed that no mutations had occurred in the P_E1 promoter region. The pLP208 insert could only be cloned into pFX3 in a way that the P_E1 promoter was in the same orientation as the chloramphenicol resistance gene. The reason for this is not known, however it could be that there is no termination site in the shortened transcript of the pLP208 insert and that the transcript interferes with plasmid replication. Directional cloning, using two different restriction enzymes could force the pLP208 insert into the opposite direction in pFX3. The plasmids which acquired mutations in the *ori*-inserts could have been re-transformed into *L. lactis*. A higher transformation frequency after the second transformation would indicate that the mutations were required in order for the *ori*-plasmids to replicate. However, time did not permit these experiments and they were also not regarded as relevant for this study.

The cause of these mutations and deletions in P_E1 may be explained by the features of the pFX3 plasmid. Plasmid pFX3 contains the pDI25 replicon, which belongs to the pE194/pLS1 replicon family (for reviews see Khan, 1997, 2000) and replicates via rolling-circle replication. It has previously been demonstrated (Kiewiet *et al.*, 1993) that theta replicating plasmids in *L. lactis* are more stable than plasmids replicating via rolling circle. An important factor in structural instability (plasmid DNA rearrangements) and segregational instability (loss of entire plasmid populations) of rolling-circle replicating plasmids is the formation of single-stranded DNA intermediates. The free single-stranded DNA is homologous to the plasmids and thereby elevates the recombination frequency (Peeters *et al.*, 1988), while the single-stranded DNA nick used for initiation of replication can also become recombinogenic (Michel and Ehrlich, 1986). Furthermore the formation of linear high-molecular-weight plasmid multimers has been implicated in both structural and segregational instability.

Therefore, the mode of pFX3 replication may have contributed to the high frequency of deletions and mutations in the transformants. The reason why the P_E1 orientation in the pFX3-*ori* plasmids appeared to be correlated with the deletions and mutations in the P_E1 promoter is not known, but may be related to the pFX3 origin of replication.

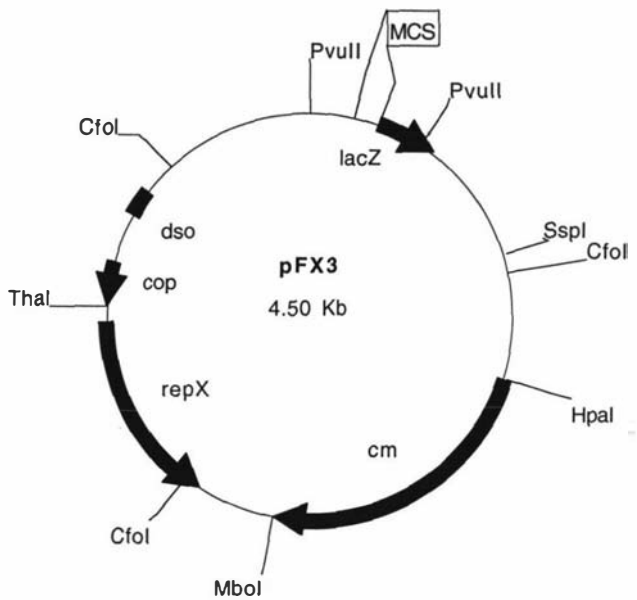


Figure 4.1. pFX3 plasmid map.
The origin of replication (cop, repx and dso [double-stranded origin]) and the chloramphenicol resistance gene are shown. The *ori*-inserts were cloned into the multiple cloning site (MCS).

Plasmid pFX3 carries the pFX2 origin (Xu *et al.*, 1991a), which has been examined previously (Xu *et al.*, 1991b). The pFX3 origin of replication contains the *cop* and *repX* genes, which are involved in replication, stability and maintenance of pFX3 (Figure 4.1; Grohmann *et al.*, 1998). The Cop protein is probably involved in copy number control and the *repX* gene encodes the replication initiator protein (Rep) (Grohmann *et al.*, 1998). The Rep protein specifically cleaves the plasmid DNA within the origin, creating a 3'-OH end, which is then elongated by the host replication machinery. It is possible that a strong promoter like P_E1 transcribing a sequence complementary to *cop* and *repX* could create antisense RNA to these genes that may lead to plasmid instability, with only plasmids containing a mutated P_E1 promoter retaining stability. Transcription from the cloned P_E1 promoter would have to go through the *lacZ* and the chloramphenicol resistance gene which

would generate a very long transcript. However, if this is true, then the chloramphenicol resistance gene should have the same effect on pFX3 plasmid replication. This would explain why the pFX3-*ori* plasmids containing P_E1 transcribing in the same orientation as *cop* and *repX* (and the opposite orientation as the chloramphenicol gene) do not carry mutations in the P_E1 promoter. Furthermore, the pFX3 *ori* is more than 2 kb from the P_E1 promoter. However, none of the transcripts detected from P_E1 were greater than 1 kb. The transcripts made from the mutated P_E1 promoters (*L. lactis* cells containing pFX3-*ori* plasmids with wildtype P_E1 in the same orientation as the chloramphenicol resistance gene were not viable) could have been detected to examine their length to test the hypothesis of antisense RNA interfering with plasmid replication. This would also show if a possible terminator downstream of the chloramphenicol resistance gene is functional. Antisense RNA of the *copx* and *rep* gene could be detected by Northern blot using a labelled single-stranded DNA or RNA probe.

The transcripts from the pFX3-*ori* plasmids were characterized by Northern blot. However, no transcripts were detected from the modified c2 *oris* that did not support pVA891 plasmid replication in *L. lactis*. The P_E1 transcripts were readily detectable in the controls pFX3-203 and pFX3-206. Although there was less P_E1 transcript detected in pFX3-206 than in pFX3-203 which is in contrast to the Northern blot using RNA from the pVA891-*ori* plasmids and might be due to the different vector system. For pFX3-207, a very large transcript was detected, presumably due to readthrough into the pFX3 plasmid from P_E1. A faint band in pFX3-203 and pFX3-206 of the same size could be seen, this band was stronger in pFX3-207, possibly due to different processing of this transcript. No transcript of the expected 207 nt could be detected. However, this transcript band could possibly have been detected using a more sensitive method, such as RNase protection, which can detect as little as 0.1 pg mRNA (Melton *et al.*, 1984). As expected, no transcripts could be detected in pFX3-204 (P_E1 deletion) and no c2 transcripts could be detected from the inverted P_E1 promoter in pFX3-213. Since the P_E1 promoter has been shown to be active (pLP201, pLP203), the transcripts made from the inverted P_E1 promoter in pFX3-213 were not detected because P_E1 (in pFX3-213) was expected to be functional. The fact that the P_E1 transcript from pFX3-207 (of the expected size) could not be detected by Northern blot, strongly suggests that it was very unstable. This is supported by the secondary structure

modelling studies of the 207 nt transcript, which only generated structures with low homology to the functional transcripts. Even small changes in the transcript sequence can result in major rearrangements in secondary structure, which in turn could impact on RNA stability. An example of this was demonstrated by Li *et al.* (2002) who showed that a single base pair mutation in $tRNA^{Trp}$ (G7→A7) results in rapid degradation of the tRNA by disrupting a GC base pair at the bottom of an acceptor stem. No proteinase transcript could be detected by Northern blot analysis from pFX3-214 (data not shown). Although the P_E1 promoter in pFX3-214 has a 1 bp mutation in the spacer region between the -10 and -35 region of P_E1 this should not have abolished P_E1 activity completely.

4.3.3 Identification of P_E1 transcript binding host proteins

The question still remains why plasmid replication is dependent on the length and sequence of the P_E1 transcript. It is already known that phage-encoded proteins are not essential for c2 origin function. Thus, it may be that the binding of lactococcal proteins to the P_E1 transcript is important for plasmid replication. Most likely candidates are host proteins that stabilize or process RNA by binding to specific structures or motifs in the transcript. To determine if lactococcal host proteins could bind to the P_E1 transcripts, electrophoretic migration shift assays (EMSA) were performed. The P_E1 transcripts were synthesized by *in vitro* transcription from a T7 promoter. It has been shown that transcripts synthesized by *in vitro* transcription are biologically active (Melton *et al.*, 1984), and as T7 polymerase has a low error rate ($6x\ 10^{-5}$, Brakmann and Grzeszik, 2001) it was highly unlikely that any mutations occurred during *in vitro* synthesis of the P_E1 transcript. In every gel shift experiment tRNA was included in the preincubation step to reduce proteins binding non-specifically to the transcripts. The gel shift experiment using the 207 nt probe detected a single RNA-protein complex, while two RNA-protein complexes were detected using the 307 nt probe. It is possible that a different number of proteins/protein-complexes bind to the two different transcripts. However, the gel shift assays did not appear to be reliable indicators of specific protein-transcript binding, as North-Western blots showed that the same set of proteins bound to the 207 nt, 273 nt, and 307 nt P_E1 transcripts and

the riboprobe kit control transcript (T7 gene 10). To eliminate the possibility that this was due to one of the steps in the North-Western blotting procedure (denaturing electrophoresis, blotting and renaturation), direct isolation of proteins by affinity purification was attempted. The T7 gene 10 codes for the capsid protein and shows no sequence similarity to the transcripts made from P_E1.

The same 44 kDa protein was isolated by affinity purification, irrespective of whether immobilized 307 nt or 207 nt transcripts were used. This protein was identified by amino-terminal amino acid sequencing and a database search as the ribosomal protein S1, although previous sequence analysis has shown that the P_E1 transcript does not contain a ribosome binding site (Lubbers *et al.*, 1998). The bacterial ribosome (70S) consists of a single, small 30S subunit and large 50S subunit. The 30S subunit consists of a 16S rRNA and 21 proteins (S1 to S21) and the 50S subunit consists of a 23S rRNA, a 5S rRNA and 33 proteins (L1 to L36). The S1 protein is only weakly bound to the 30S ribosomal subunit and its major function is to bind mRNA and thus facilitate protein synthesis (for review see Subramanian, 1983).

The *E. coli* S1 protein (557 aa) contains six S1 domains (repeat motifs). The four C-terminal domains are highly conserved and bind RNA (Schnier and Isono, 1982), while the two N-terminal domains are divergent and bind to the ribosome (Giorginis and Subramanian, 1980). The S1 domain was originally identified in the S1 protein but it is also found in PNPase, RNase E, NusA (transcription factor), RNase II, PRP22 (RNA helicase) and many other RNA binding proteins (Bycroft *et al.*, 1997).

The structure of the S1 domain of PNPase has been solved by NMR (nuclear magnetic resonance, Bycroft *et al.*, 1997) and contains a 5-stranded antiparallel β barrel, an α helix and a 3_{10} helix. The RNA-binding site is thought to be formed by conserved residues on one face of the barrel and the adjacent loops (Bycroft *et al.*, 1997).

Amino acid sequence alignment of the S1 proteins of *E. coli* and *L. lactis* showed that the proteins have 32% sequence identity. Multiple sequence alignment of the S1 domain of PNPase and the S1 proteins of *E. coli*, *L. lactis* and three other bacteria revealed two conserved S1 domains in the S1 protein of *L. lactis* (Figure 4.2). The conserved residues that are thought to be involved in RNA binding, are also shown for all five S1 proteins.

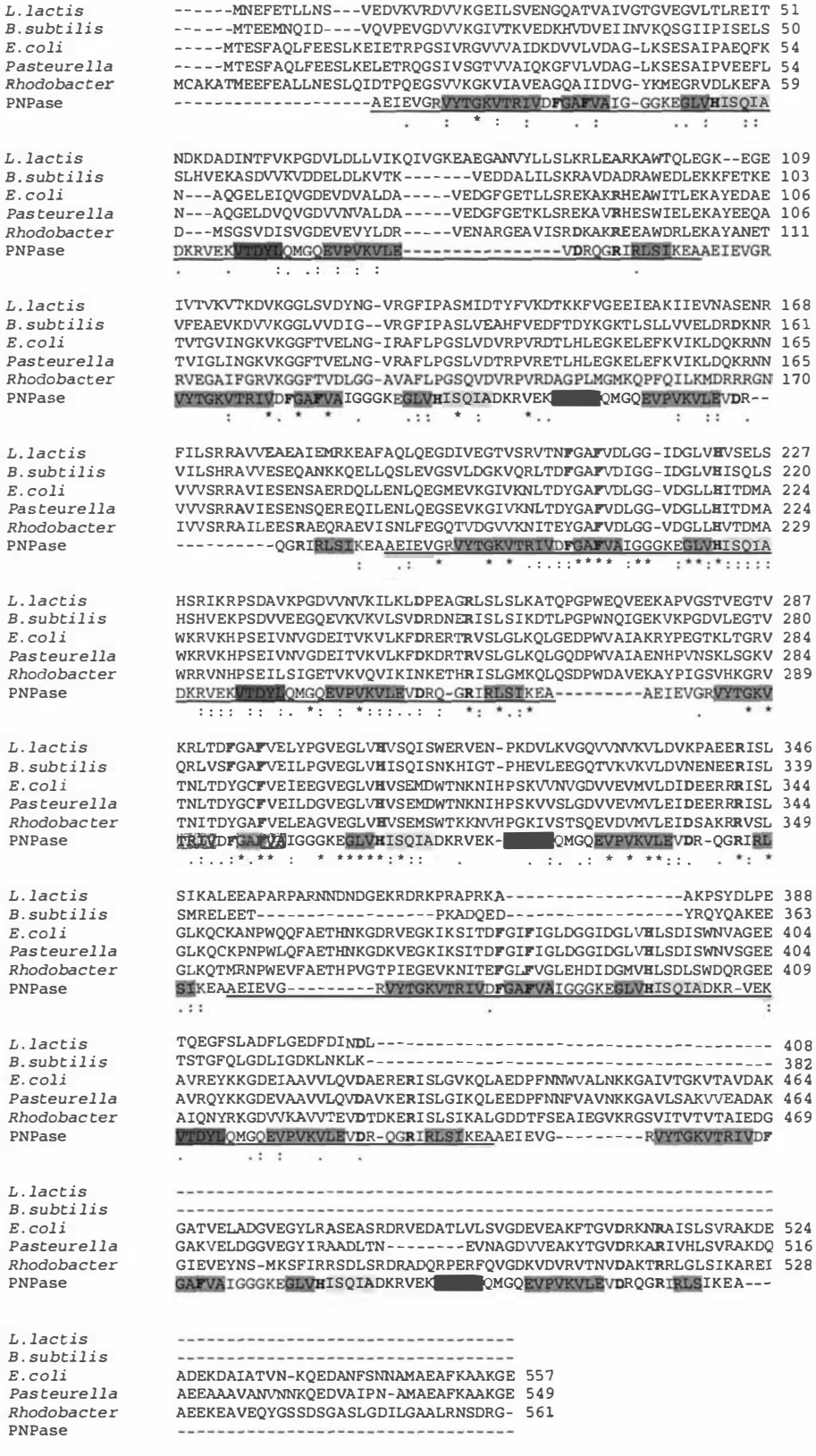


Figure 4.2. Multiple sequence alignment of the ribosomal protein S1 using the program ClustalW.
The sequence containing the PNPase S1 domain has been repeated six times (first, third and fifth repeat sequences are underlined) and then aligned with the ribosomal proteins S1 of *L. lactis*, *B. subtilis*, *E. coli*, *Pasteurella* and *Rhodobacter*. The bold letters show residues, which are believed to be involved in RNA binding. The shaded sequences represent the five β strands (), the α helix () and the 3_{10} helix (). The · represents conserved changes, the : highly conserved changes, and the * identical amino acids.

Protein S1 is not required for assembly of the 30S ribosomal subunit (Held *et al.*, 1973) but it is essential for the initiation of translation for most mRNAs in *E. coli* (Sørensen *et al.*, 1998). As the S1 protein is the only ribosomal protein that has a high affinity for mRNA (Sengupta *et al.*, 2001), it is possibly not surprising that S1 bound to the P_E1 transcripts in the affinity purification experiments. However, S1 has also been shown to interact with *E. coli* poly(A) tails and degradosome proteins such as PNPase and RNase E (Feng *et al.*, 2001). Thus, the S1 protein may link the processes of mRNA degradation and translation.

Binding experiments with artificially created RNAs show that S1-depleted 30S subunits bind RNAs containing a Shine-Dalgarno sequence whereas intact 30S subunits and purified S1 protein both preferentially bind RNAs that contain a potential pseudoknot structure (Ringquist *et al.*, 1995). The S1 binding motif contains a pseudoknot with highly conserved sequence elements (see Figure 1.8; Bourdeau *et al.*, 1999). Interestingly, a long-range pseudoknot in the RNA of RNA phage Qβ is essential for Qβ genome replication (Klovins and van Duin, 1999). Protein S1 is part of the RNA phage Qβ replicase (Blumenthal and Carmichael, 1979), which also contains a phage encoded subunit (polymerase activity), elongation factors EF-Tu and EF-Ts, and a host factor (HF). Protein S1 allows the replicase to bind to specific binding sites in the RNA (Miranda *et al.*, 1997). However, it has been shown in other binding studies that S1 can also bind to poly(U) and pyrimidine-rich sequences in Qβ RNA (Brown and Gold, 1995). Although the program PKnots did not find any potential pseudoknots in the modelled P_E1 transcript structure, visual examination of the modelled secondary structure of the 273 nt transcript revealed possible base pairing between a stem-loop structure and a complementary single-stranded sequence close to the stem-loop (Figure 4.3), which corresponds to the classic description of a pseudoknot (for review see Hermann and Patel, 1999). In this model, positions 252 to 258 in the 273 nt P_E1 transcript are protected against ssRNA specific nuclease degradation. This could explain the 260 nt major band in the RNase protection experiment.

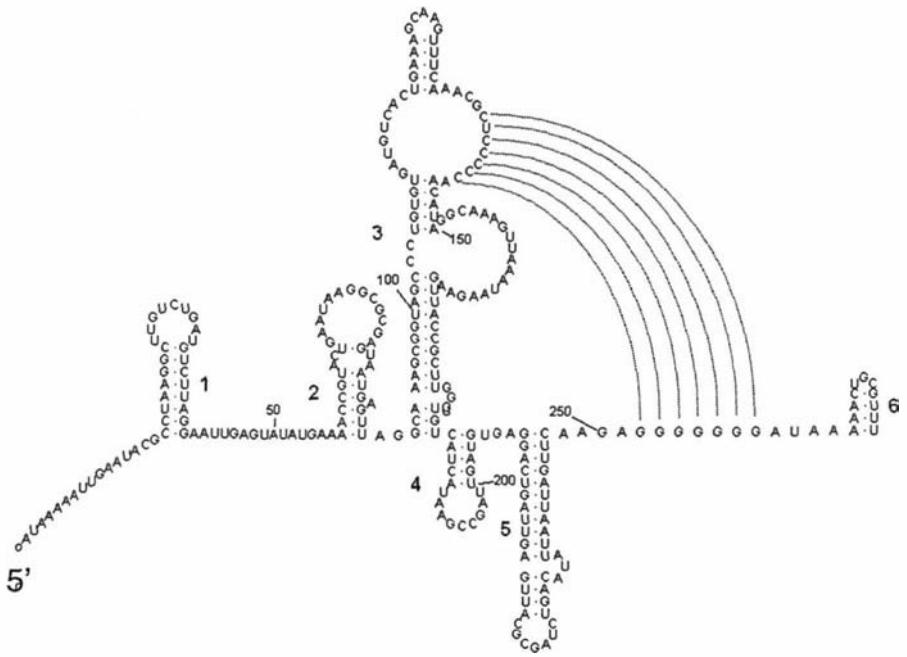


Figure 4.3. The proposed tertiary structure (pseudoknot) of the 273 nt transcript from pLP206. The numbers one to six indicate different stem-loop structures.

In the North-Western blots the S1 protein bound to all P_E1 transcripts and to the control transcript (T7 gene 10). Binding of the 30S ribosomal subunit to RNA via the S1 protein may be an important mechanism for preventing degradation of RNA by ribonucleases. This is supported by the findings of Agaisse and Lereclus (1996) who demonstrated that a Shine-Dalgarno sequence in the 5' untranslated region of *cryIII A* mRNA in *Bacillus thuringiensis* is important for mRNA stability. To test the hypothesis that S1 is involved in c2 replication, an S1 knockout mutant or an *in vitro* replication assay would be required to examine the ability of c2 to replicate in the presence and absence of S1. However, a *L. lactis* knockout strain of S1 is likely to be lethal, because S1 is an absolute requirement for translation of all mRNAs in *E. coli* (Sørensen *et al.*, 1998) and an *in vitro* system has yet to be established in *L. lactis*.

Although the 44 kDa protein was identified as the ribosomal protein S1 by N-terminal sequencing, identification of the 25 kDa protein proved to be more difficult, as an unambiguous amino-terminal sequence of this protein could not be obtained. The

best sequence matches were to the 50S ribosomal protein L4 (22.3 kDa) and the 30S ribosomal protein S4 (23.1 kDa). It is possible that the 25 kDa protein seen on the 12% SDS gel after affinity purification actually consisted of two protein bands that were poorly resolved. The other five protein bands seen in the North-Western blots could not be detected by the affinity purification possibly due to the complex nature of the north-western blotting procedure (see page 121).

4.3.4 Comparison of plasmid DNA per cell and the amount of P_E1 transcript produced

The amount of plasmid DNA isolated from *L. lactis* containing the various pVA891-*ori* plasmids varied considerably, suggesting that the different plasmids had different copy numbers per cell. The plasmid copy number of the pVA891-*ori* plasmids were calculated from the results in Figure 3.16.

There are different mechanisms of erythromycin resistance which include target site alteration, antibiotic modification, and altered antibiotic transport (Weisblum, 1995). Plasmids pVA891 and pTRKL2 both carry erythromycin resistance genes (*ermB* and *ermAB* respectively; Macrina *et al.*, 1983; O'Sullivan and Klaenhammer, 1993) that encode an adenine-specific *N*-methyltransferase, which alters the antibiotic target site. This enzyme methylates 23S rRNA and thereby prevents binding of erythromycin to the 50S ribosomal subunit; erythromycin inhibits peptide chain elongation. The results from the plasmid copy number analysis support previous reports that a very low copy number plasmid carrying the gene for the methylase is sufficient to establish erythromycin resistance (Horodniceanu *et al.*, 1976).

The growth rates of the lactococcal strain MG1363 containing the various pVA891-*ori* plasmids showed no statistically significant difference when compared to each other or the pTRKL2 control. This was also the case for strain NZ9800 containing pLP212 in the presence or absence of nisin or pTRKL2. The similarity of the growth curves indicated that the plasmids were neither a burden for their host or an advantage. However, strain NZ9000 containing pLP212 in the absence of nisin showed a statistically significant lower growth rate compared to NZ9000 containing pLP212 in

the presence of nisin, pTRKL2 or plasmid free. The low copy number of pTRKL2 in NZ9000 in the absence of nisin (table 4.1) might be responsible for the slower growth of this strain.

For pAMβ1, replication is limited by transcription passing through the origin in the same direction as replication (Bruand and Ehrlich, 1998). However, this did not appear to be the case for the pVA891-*ori* plasmids as there was no correlation between the amount of P_E1 transcript and the amount of plasmid DNA per *L. lactis* cell (Figure 4.4), nor was there a correlation between the amount of P_E1 transcript and the electroporation frequency.

The amount of transcript and plasmid DNA detected in pLP205 was low (see section 4.3.1) compared to pLP201 and pLP203, and may be the result of decreased transcription and therefore decreased plasmid replication. In pLP206 the amount of transcript detected was almost as high as in pLP201 and pLP203, but the amount of plasmid DNA detected was very low. This could be due to a more stable transcript in pLP206 compared to the longer transcript in pLP201 and pLP203. This is supported by secondary structure modelling of the transcript from pLP206 which showed a stem-loop structure at the extreme 3' end. In addition, the transcript from pLP206 showed the potential to fold into a tertiary structure which may also confer some protection against nuclease degradation.

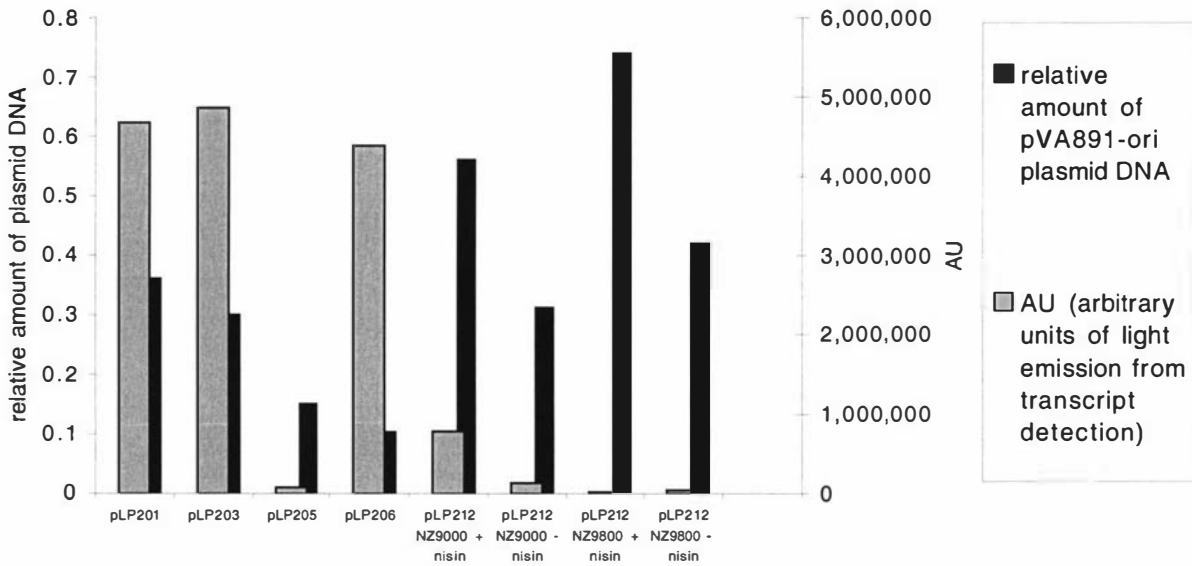


Figure 4.4. Correlation between the calculated amount of plasmid DNA (see Figure 3.18) and the calculated amount of P_E1 transcript detected by northern blot analysis (see Figure 3.10).

The amount of P_E1 transcript detected for pLP212 (in NZ9000 and NZ9800) was low (section 4.3.3) compared to the pVA891-*ori* plasmids in MG1363. The amount of plasmid DNA detected for pLP212 was always higher in the presence of nisin than in its absence for both the NZ9000 and NZ9800 strains, which was also consistent with the amount of P_E1 transcript isolated. However, the overall amount of DNA isolated from pLP212 was notably higher than the amount of DNA isolated from the other pVA891-*ori* plasmids. Plasmid pTRKL2 was used as a control for plasmid isolation in all three strains. As the amount of isolated pTRKL2 DNA was similar for these strains, this indicated that the differences in the amount of plasmid DNA in the pLP-plasmid series was functional rather than strain related.

4.3.5 Phage c2 depends on lactococcal proteins for replication

The results from the experiment to determine if c2 confers a Per phenotype (phage encoded resistance) were unambiguous. When c2 was plated on lawns of MG1363 cells containing pFX3-*ori* plasmids, there was no change in plaque morphology, timing of plaque appearance or a decrease in titer indicating that the presence of the phage *ori* on a high copy number plasmid did not titrate out any phage proteins required for replication. This suggests that c2 relies completely on host proteins for its replication. In contrast to c2, BK5-T (Mahanivong *et al.*, 2001) and all of the P335 phages examined to date (ϕ50 (Hill *et al.*, 1990b), ϕ31 (O'Sullivan *et al.*, 1993), Tuc2009, Q30, Q33, ul36 (McGrath *et al.*, 2001), and TP901-1 (Østergaard *et al.*, 2001)) confer a Per phenotype. It has been demonstrated for some of these cloned origins that they do not support plasmid replication in *L. lactis* when cloned into a plasmid which lacks an origin of replication for this bacteria (ϕ31, Madsen *et al.*, 2001; BK5-T, Mahanivong *et al.*, 2001). This presumably indicates that the P335 phages require phage encoded proteins for phage replication and is in contrast to the c2 *ori,* which can support plasmid replication in *L. lactis* in the absence of phage proteins.

In addition to the cloned c2 origin, plasmid replication in *L. lactis* is also supported by the cloned origin of phage sk1, which shows a similar *ori* organization to phage c2. The cloned origin of phage sk1 contains 179 codons from the N-terminus of ORF 47, the intergenic region between ORFs 47 and 48 (containing 3 direct repeats),

and the early promoter 1 (P_E1, Figure 4.5; Chandry *et al.*, 1997). However, it is unknown if expression of the truncated ORF sequence is required for plasmid replication and there is no data currently available on whether sk1 exhibits a Per phenotype.

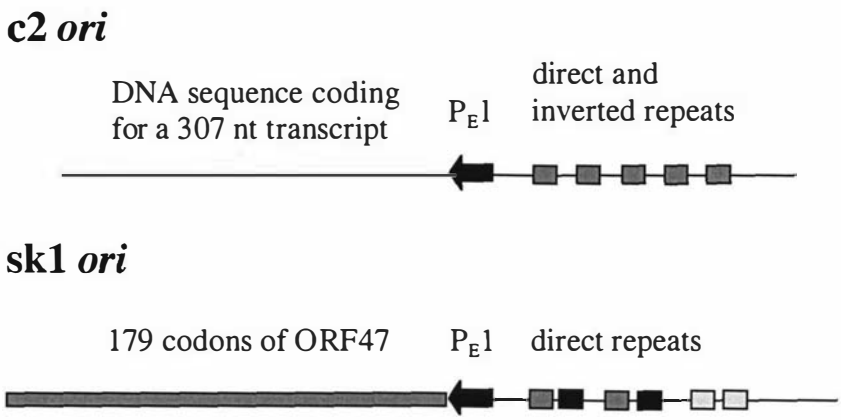


Figure 4.5. Schematic diagram of the c2 and sk1 origins of replication, which support plasmid replication in *L. lactis*.

Figure reproduced and modified from Waterfield *et al.*, 1996 and Chandry *et al.*, 1997 (not drawn to scale).

Most of the known bacteriophages require both host and phage-encoded proteins for DNA replication. One notable exception is phage phi U1, which depends completely on host proteins for replication. The cloned origin of DNA replication of phi U1 supports plasmid replication in *Streptomyces lividans* when cloned into a plasmid lacking a functional Gram-positive *ori*. The absence of an open reading frame in the phi U1 *ori* fragment indicates that only host factors are required to initiate replication at the phi U1 origin of DNA replication (Farkasovská *et al.*, 2001). Although it is quite unusual for a phage to rely completely on host proteins for DNA replication, the results in this thesis suggest that phage c2 may represent a second example of a phage which is completely dependent on the host cell for its replication.

4.4 Future studies

The results presented in this study should facilitate further research focusing on the importance of the P_E1 transcripts in replication and any host proteins associated with the transcripts.

The work described in this thesis has already formed the basis for continued research on the origin structure in different prolate-headed lactococcal phages. Rakonjac *et al.* (2002, submitted) found only three distinct groups of origins in 122 prolate-headed lactococcal phages: the bIL67 group, the c2 group, and the 923 group. There is very little similarity in the P_E1 transcripts (13% identity) between the different origin groups, although they are highly conserved within a group (95% identity). Despite the sequence divergence, each origin could support replication of pVA891 in all of the host *L. lactis* strains for the various phage (MG1363 for c2, IL1403 for bIL67, and 112 for 923), which suggests that the origins are functional in all tested hosts. However, origin screening plasmids (pVA891) carrying a chimeric origin (3' end of transcript from 923 and 5' end of transcript and promoter from c2, and vice versa) did not replicate in *L. lactis,* indicating that the P_E1 transcript sequence is critical for phage replication. In addition, the length and abundance of the P_E1 transcripts synthesized by the three phages were examined by RNase protection analysis. In this experiment multiple transcript bands were detected for all three phages, indicating that the transcripts are processed by RNA processing enzymes (Rakonjac *et al.*, 2002, submitted).

The results presented in this thesis also suggest that the transcripts synthesized from P_E1 may be processed by RNA processing enzymes. In ColE1 replication, the DNA-RNA hybrid is cleaved by RNaseH (*rnh*). The cleaved transcript serves then as a primer for initiation of DNA replication by DNA polymerase I (*polA*), and replication is completed by DNA polymerase III holoenzyme. However, ColE1 can also replicate in *rnh polA* and *rnh polA*$^+$ bacteria, but not in *rnh*$^+$ *polA* bacteria (Dasgupta *et al.*, 1987). Thus the uncleaved transcript must either serve as a primer, or else the DNA-RNA hybrid allows assembly of the primosome on the single-stranded DNA strand. In contrast, plasmid pAMβ1 does not transform *polA Bacillus subtilis* cells. As plasmid pLP203 can replicate in *polA L. lactis* cells (Rakonjac, unpublished results), the phage

c2 origin must be able to initiate DNA replication by a mechanism that does not require DNA polymerase I. It would be interesting to know if the pVA891-*ori* plasmids can also replicate in *rnh L. lactis* cells or other *L. lactis* bacteria deficient in certain RNA processing enzymes or proteins involved in replication. This would be a valuable first step for determining the host enzymes that are essential for c2 replication in *L. lactis.*

The results from the current study indicated that the non-functional P_E1 transcripts are probably unstable and rapidly degraded. Additional experiments like RNA pulse-chase would substantiate this observation, and allow accurate determination of the half-life of these putatively unstable RNA molecules (Belasco *et al.*, 1985). However, adapting the pulse-chase experiment for Gram-positive bacteria involves considerable technical challenges. A simpler alternative would be to utilize rifampicin, which binds specifically to the β subunit of bacterial RNA polymerases and prevents transcription initiation. By adding rifampicin to the cells and then quantifying the P_E1 transcripts by Northern blot analysis, the half-life of the P_E1 transcripts could be determined (Bernstein *et al.*, 2002).

To improve the secondary structure models of the P_E1 transcripts, the length of the processed transcripts has to be determined with greater accuracy. This could be achieved by performing RNase or S1 nuclease protection experiments (Sambrook *et al.*, 1989) using a shorter probe. In addition, mutations and deletions of sequence elements in the transcript could be created that would disrupt the predicted secondary structures. This would give valuable information on the importance of the individual structural elements in plasmid replication.

Most replication origins contain AT-rich sequences that function as DNA-unwinding elements (DUEs), and which are important in origin DNA unwinding. As the highly conserved AT-rich region in the c2 origin is not required for replication, the sequence downstream of P_E1 may function as a DUE. Nuclease sensitivity experiments would assist identification of the region that functions as a DUE. In these experiments increased sensitivity to ssDNA specific nucleases is used to detect helical instability from a potential DUE within supercoiled plasmid DNA. Mung bean nuclease nicks supercoiled plasmid substrates in unwound regions (Kowalski and Sandford, 1982) and could be used in this study.

To answer the question of whether formation of a stable DNA-RNA hybrid in the origin is important for replication, the presence of a stable hybrid could be assayed by potassium permanganate treatment followed by primer extension analysis. Potassium permanganate oxidizes pyrimidine bases within ssDNA regions, thus permanganate hypersensitivity on the non-template strand in a specific region could be used to identify the formation of a persistent RNA-DNA hybrid (Sasse-Dwight and Gralla, 1989).

Many bacterial and phage origins have been examined in plasmid systems. In order to facilitate manipulation of the c2 origin, all the experiments concerning c2 DNA replication in this study have also been performed in a plasmid system. However, it is not known if the requirements for DNA replication at the c2 *ori* in a plasmid are the same as at the c2 *ori* in the context of the larger phage genome. To substantiate the findings of this study, the various mutations will need to be created and analyzed in phage c2 itself.

As shown in a previous publication (Waterfield *et al.*, 1996) and also demonstrated in this thesis, only host encoded proteins are required for phage c2 DNA replication in the plasmid system. It would be of great value to develop an *in vitro* replication system (Crooke, 1995) for determining which host proteins are involved in c2 replication. In such a system, several host proteins known to be involved in DNA replication could be examined for their ability to bind to the c2 origin and for their involvement in c2 replication using DNA footprinting experiments. However, the implementation of this study is complicated by the current lack of an *in vitro* replication system in *L. lactis*.

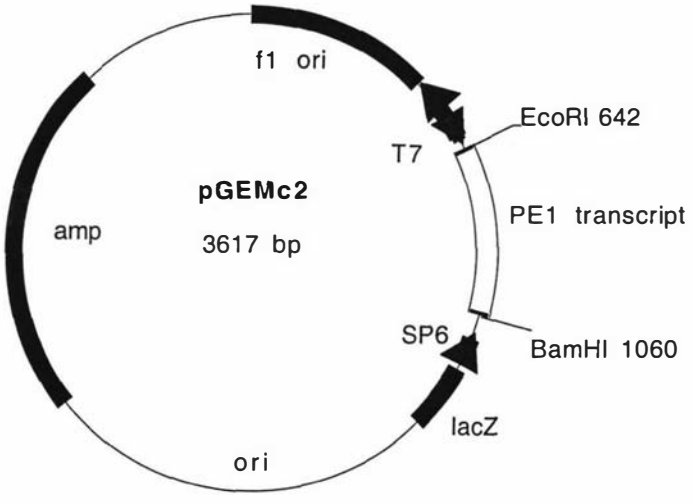
Bacteria and phages have evolved side by side, bacteria always trying to fight phage infection by developing resistance mechanisms while at the same time phages try to overcome these mechanisms and take advantage of their hosts. A detailed understanding of the mechanisms of prolate phage DNA replication and interactions with host replication machinery would provide a powerful model to understand the co-evolution of phage and their hosts.

Appendix 1. Physical maps of the plasmids used in this study.

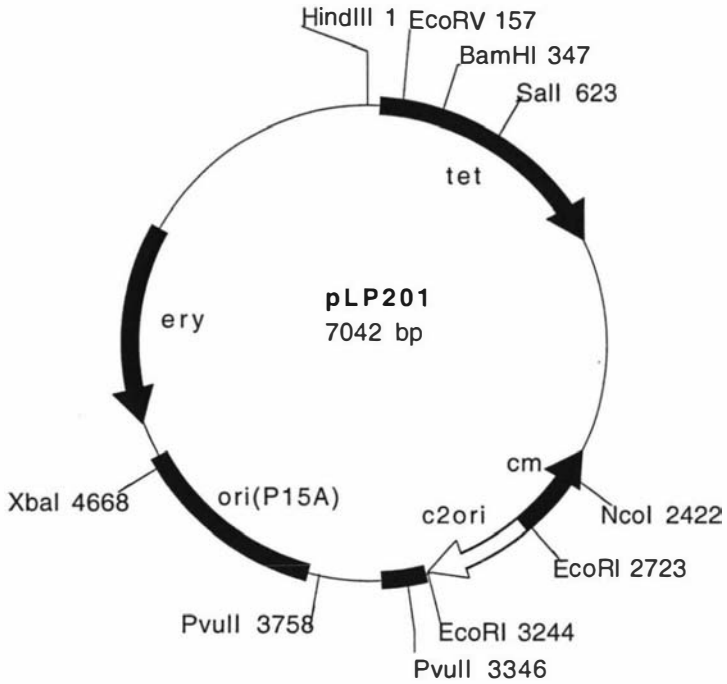
The following plasmid maps illustrate vector sizes, genetic elements and their orientation, origins of replication and multicloning sites of the plasmids used in this study. See Table 2.5 for the source of plasmids.

The maps also show restriction sites which were important for cloning processes or probe preparations during the presented studies. The orientation of the c2 *ori* fragments is shown as the orientation of the P_E1 promoter, except for pFX3-213 (P_E1 inversion), here the orientation of the DNA sequence coding for the transcript is shown.

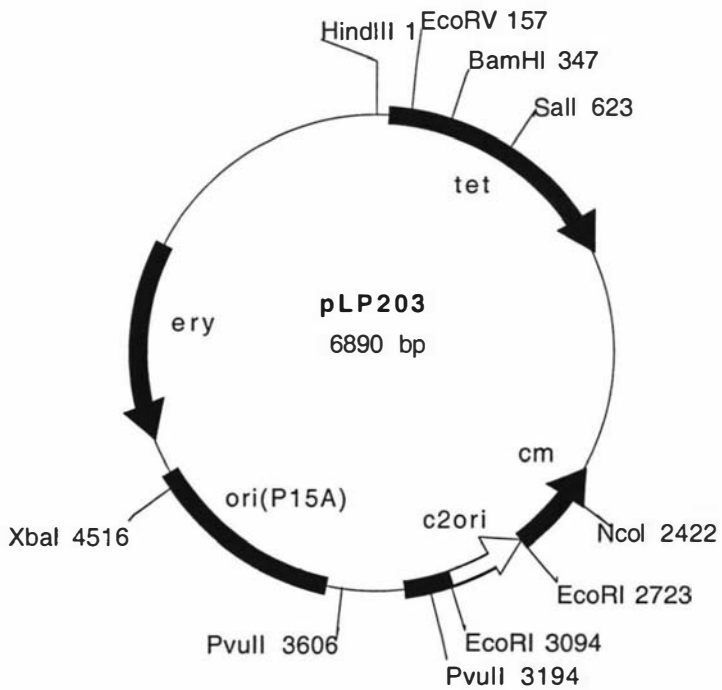
A1.1 Plasmid pGEMc2. 418 bp DNA sequence coding for the P_E1 transcript from c2 cloned downstream of the T7 promoter of pGEM®-3Zf.



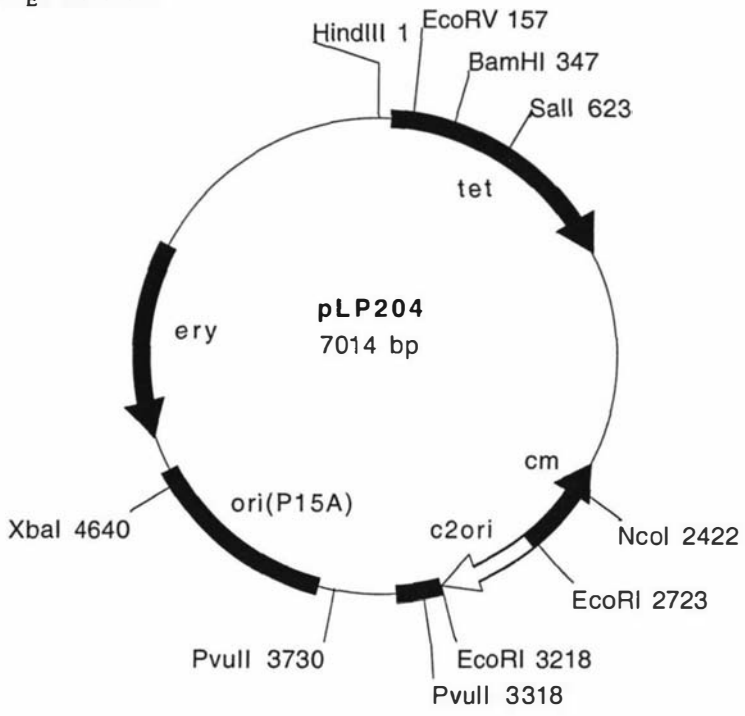
A1.2 Plasmid pLP201. 521 bp *ori* fragment from c2 cloned into pVA891, containing P_E1 and P_L1.



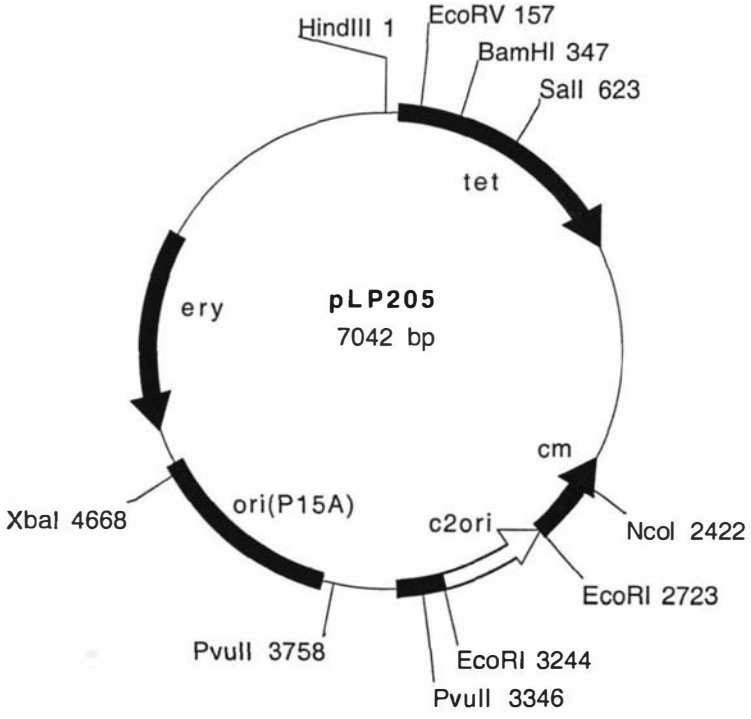
A1.3 Plasmid pLP203. 370 bp *ori* fragment from c2 cloned into pVA891, based upon pLP201, but P_L1 deleted.



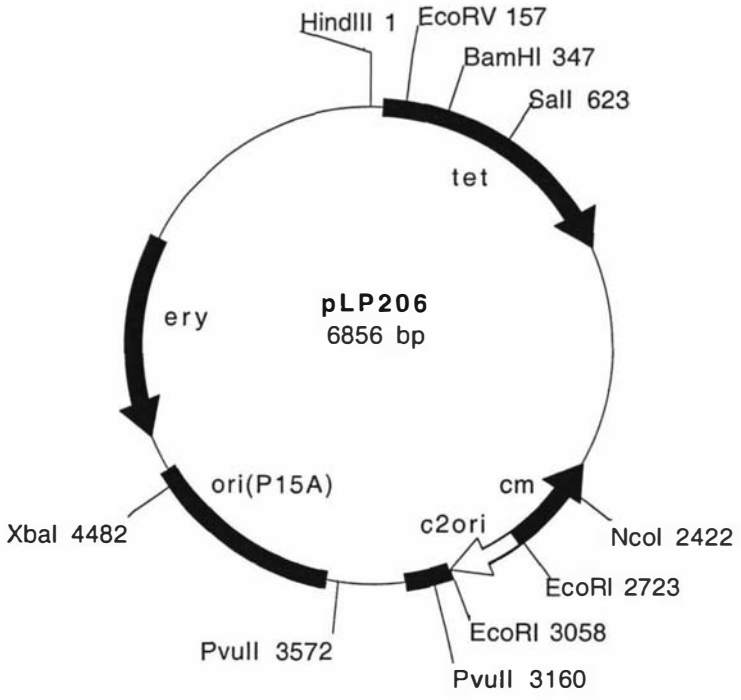
A1.4 Plasmid pLP204. 495 bp *ori* fragment from c2 cloned into pVA891, based upon pLP201, but P_E1 deleted.



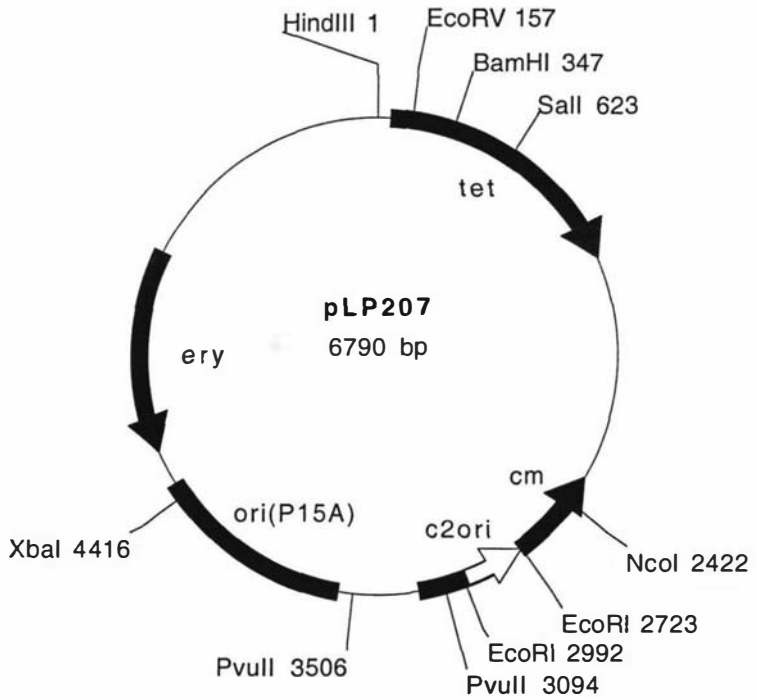
A1.5 Plasmid pLP205. 521 bp *ori* fragment from c2 cloned into pVA891, based upon pLP201, two base pair mutations in -10 region of P_E1 (TATAAT → TAGAAG).



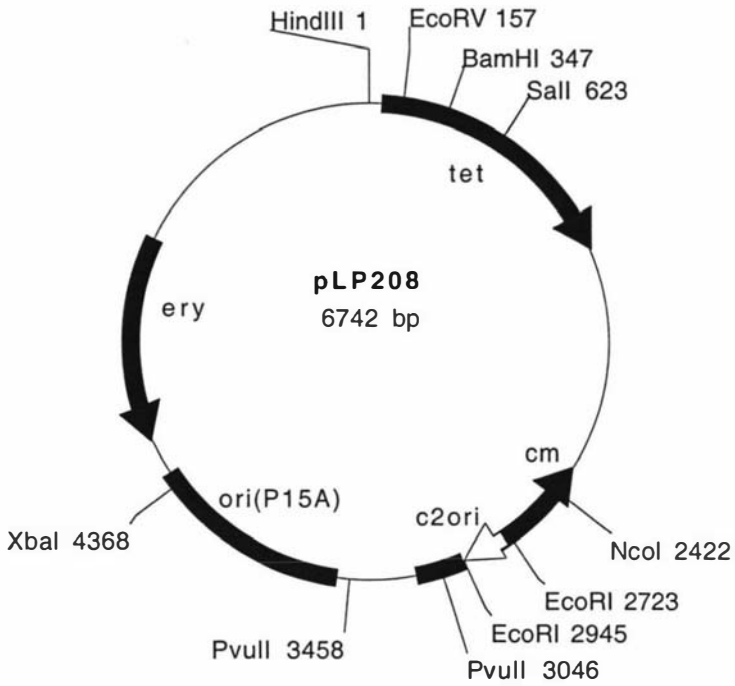
A1.6 Plasmid pLP206. 335 bp *ori* fragment from c2 cloned into pVA891, based upon pLP203, but shortened transcript.



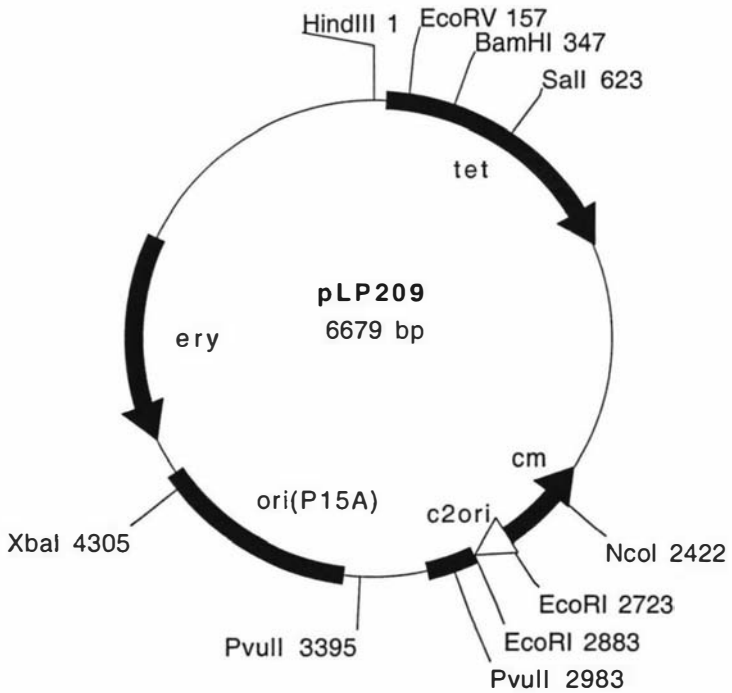
A1.7 Plasmid pLP207. 269 bp *ori* fragment from c2 cloned into pVA891, based upon pLP203, but shortened transcript.



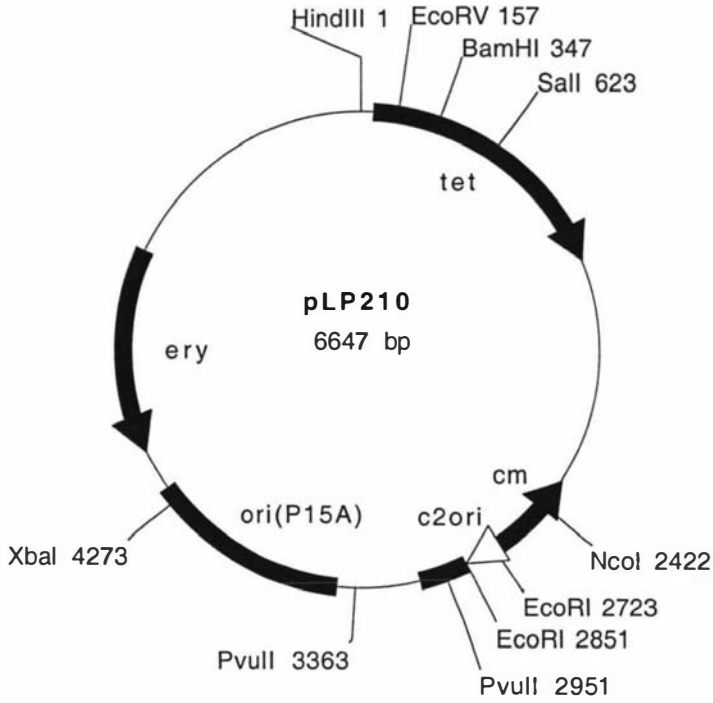
A1.8 Plasmid pLP208. 221 bp *ori* fragment from c2 cloned into pVA891, based upon pLP203, but shortened transcript.



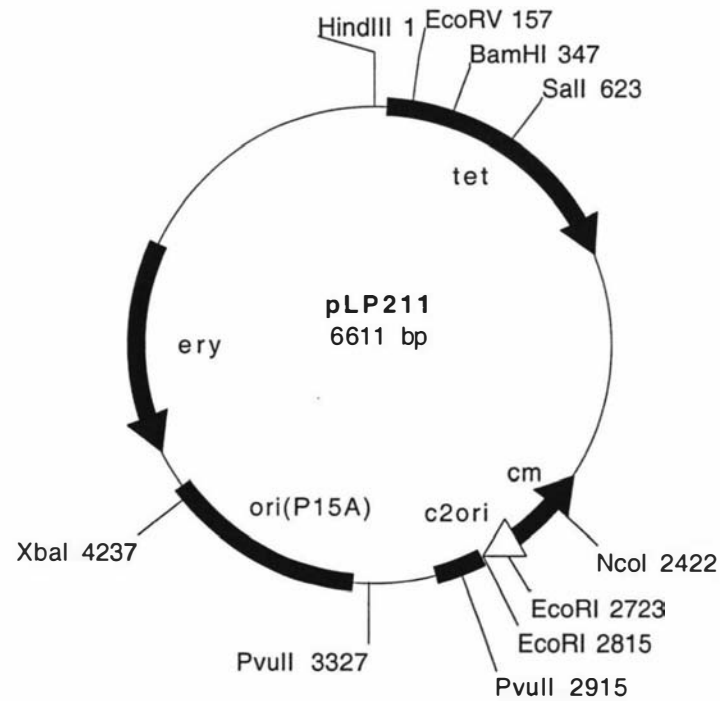
A1.9 Plasmid pLP209. 158 bp *ori* fragment from c2 cloned into pVA891, based upon pLP203, but shortened transcript.



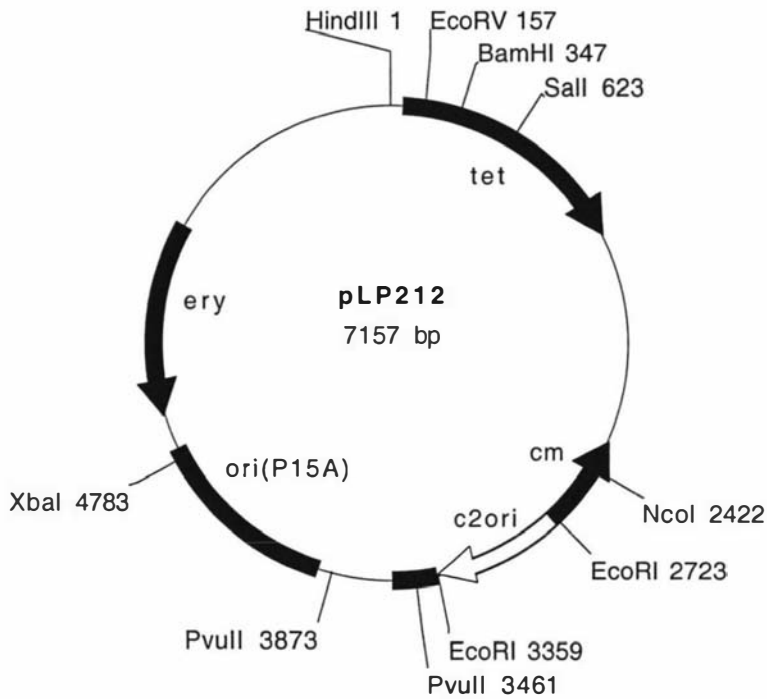
A1.10 Plasmid pLP210. 126 bp *ori* fragment from c2 cloned into pVA891, based upon pLP203, but shortened transcript.



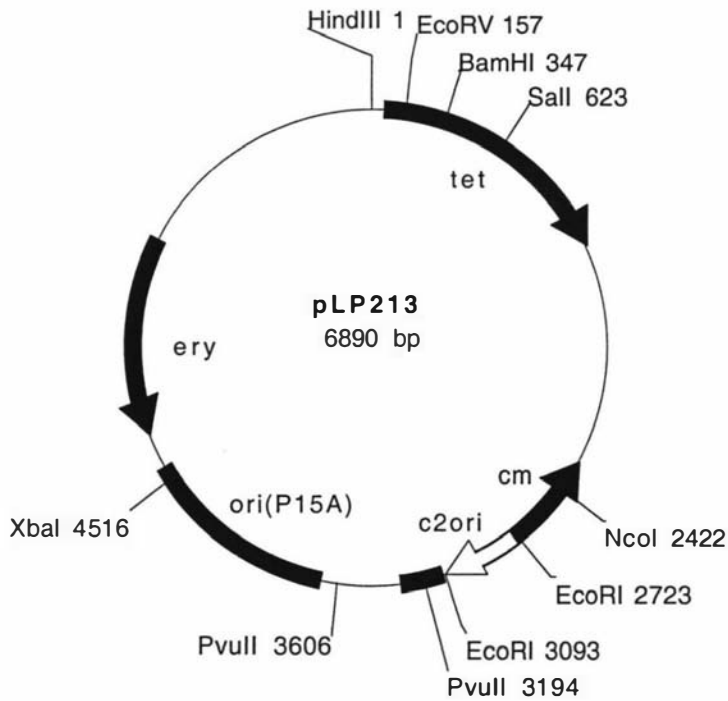
A1.11 Plasmid pLP211. 90 bp *ori* fragment from c2 cloned into pVA891, based upon pLP203, but shortened transcript.



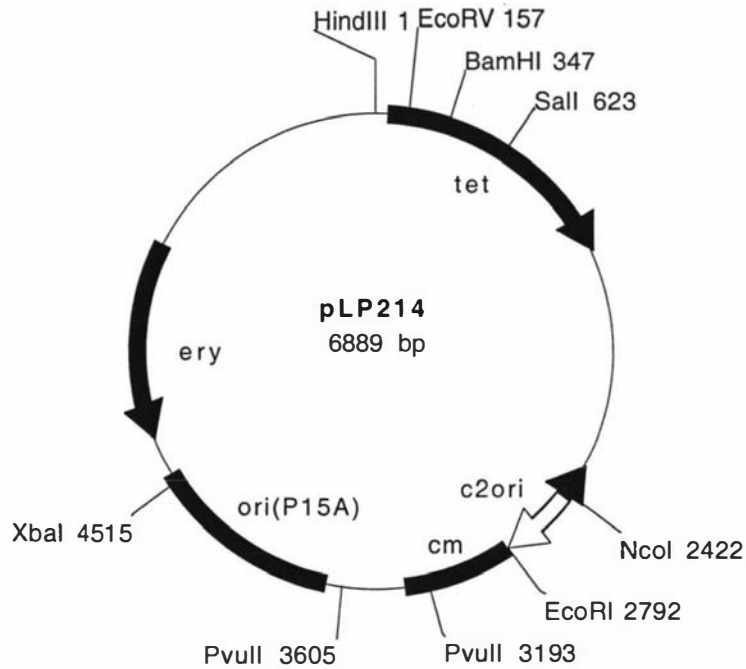
A1.12 Plasmid pLP212. 636 bp *ori* fragment from c2 (including 143 bp of *nisA* promoter) cloned into pVA891, based upon pLP201, P_E1 replaced by *nisA* promoter.



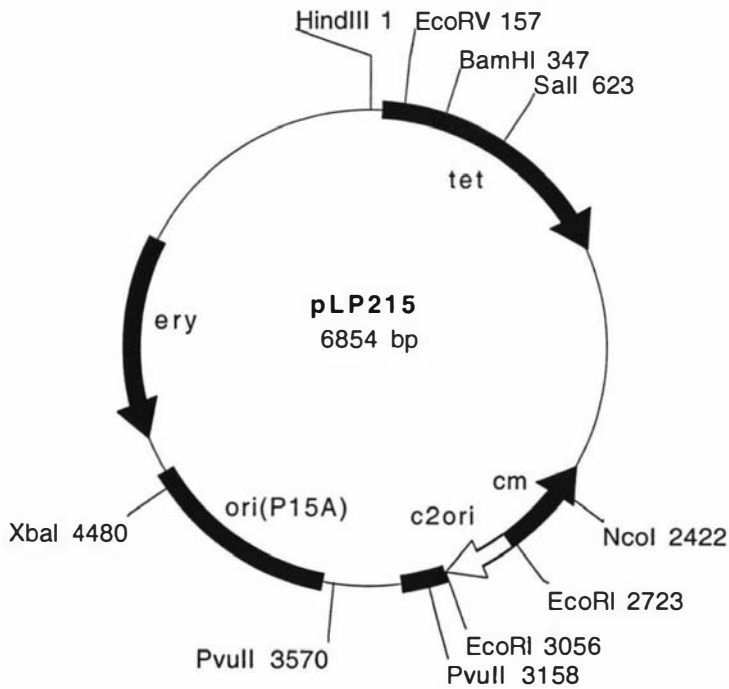
A1.13 Plasmid pLP213. 370 bp *ori* fragment from c2 cloned into pVA891, based upon pLP203, P_E1 inverted.



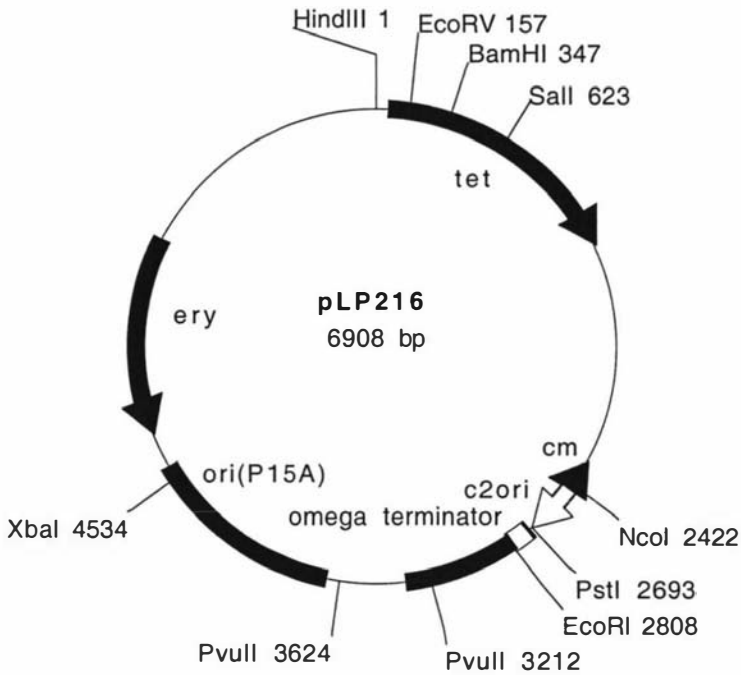
A1.14 Plasmid pLP214. 369 bp *ori* fragment from c2 (72 bp from c2, 297 bp from the *prtP* gene cloned into pVA891), based upon pLP203, but P_E1 transcript replaced by *prtP* sequence.



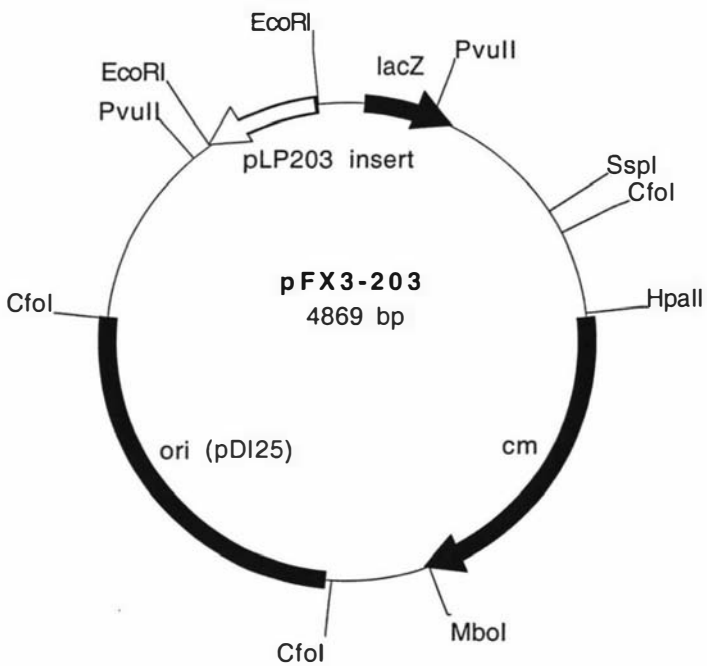
A1.15 Plasmid pLP215. 333 bp *ori* fragment from c2 cloned into pVA891, based upon pLP203, seven G-residues at 3' end of P_E1 transcript deleted.



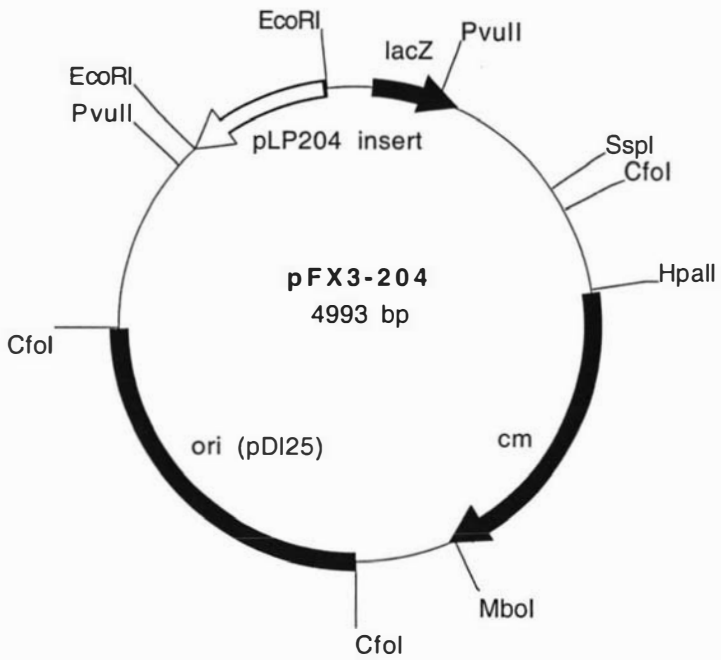
A1.16 Plasmid pLP216. 269 bp *ori* fragment from c2 and 112 bp from Ω terminator cloned into pVA891, based upon pLP207, containing the Ω terminator at the 3' end of P_E1 transcript.



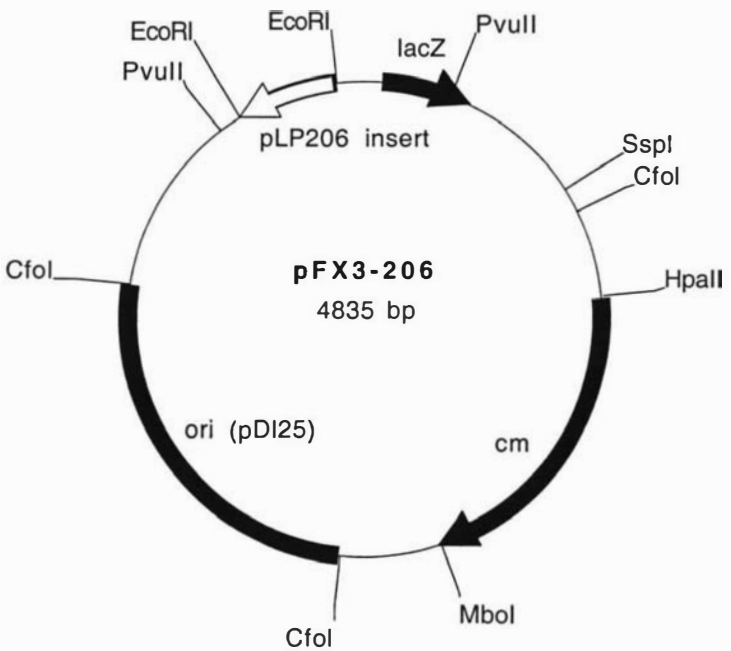
A1.17 Plasmid pFX3-203. 370 bp c2 *ori* fragment from pLP203 cloned into pFX3.



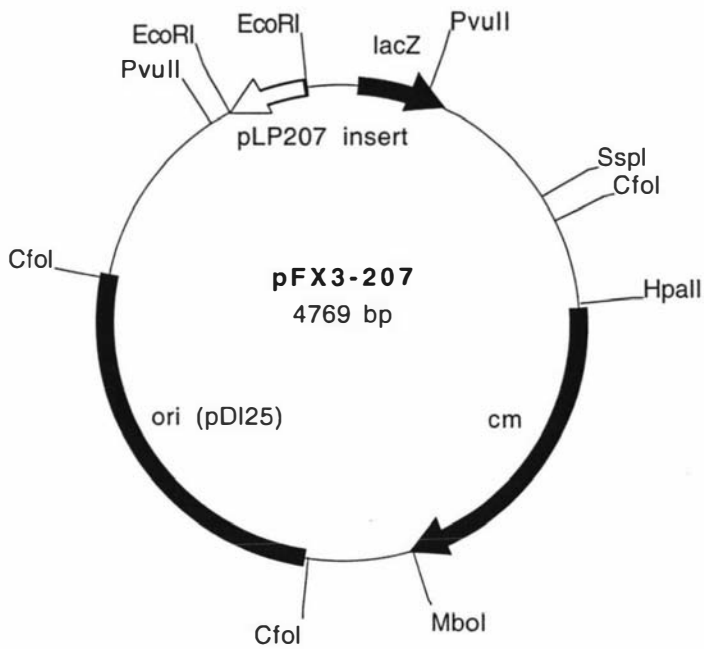
A1.18 Plasmid pFX3-204. 495 bp c2 *ori* fragment from pLP204 cloned into pFX3.



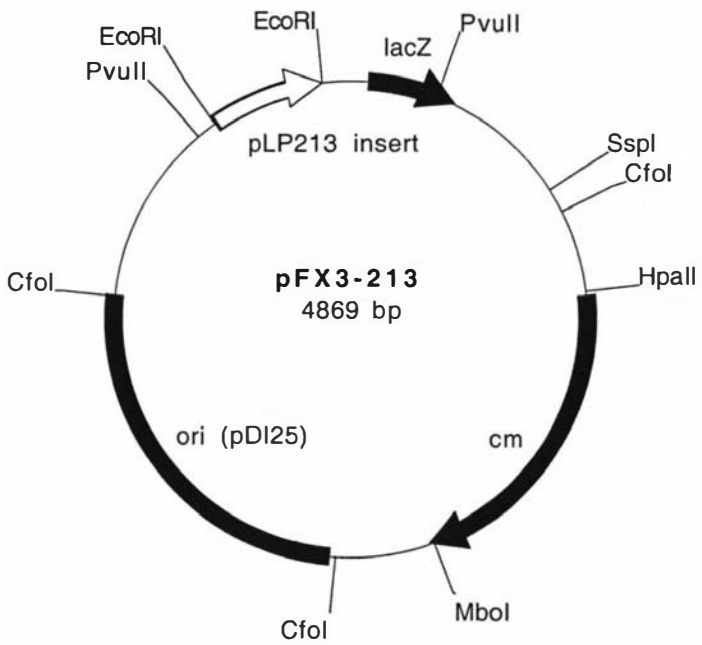
A1.19 Plasmid pFX3-206. 335 bp c2 *ori* fragment from pLP206 cloned into pFX3.



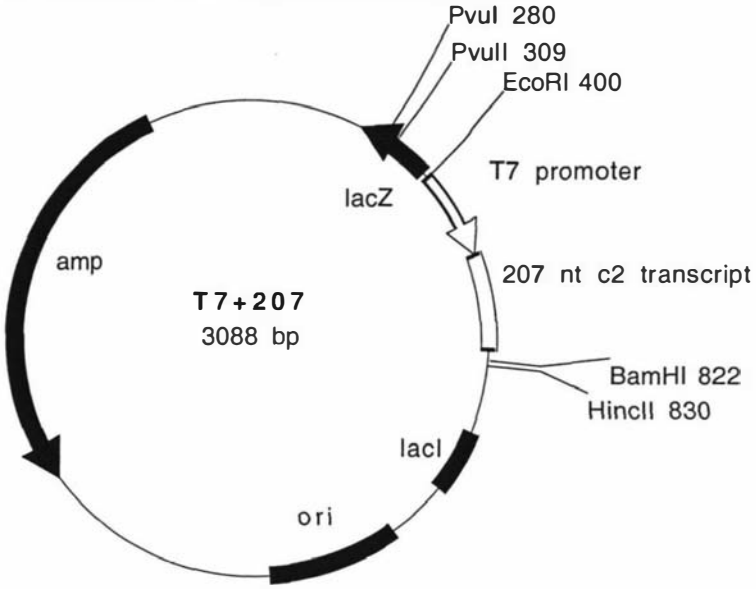
A1.20 Plasmid pFX3-207. 269 bp c2 *ori* fragment from pLP207 cloned into pFX3.



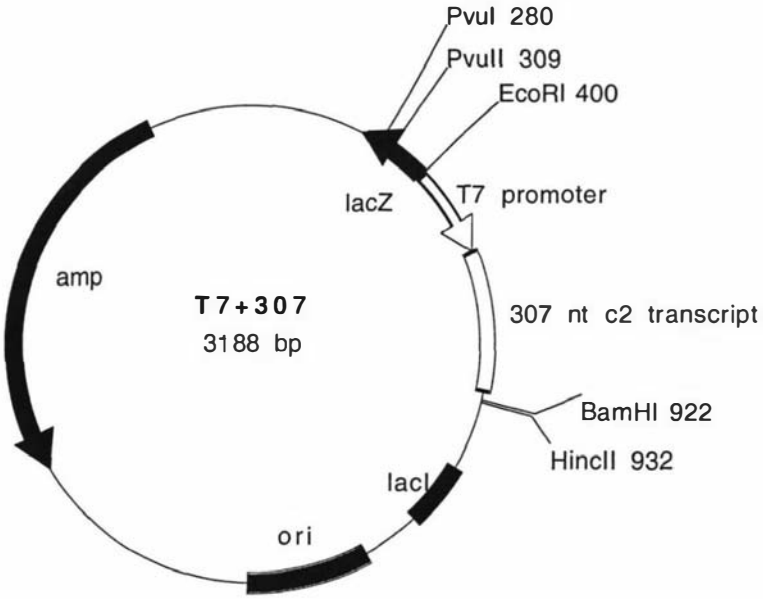
A1.21 Plasmid pFX3-213. 370 bp c2 *ori* fragment from pLP213 cloned into pFX3.



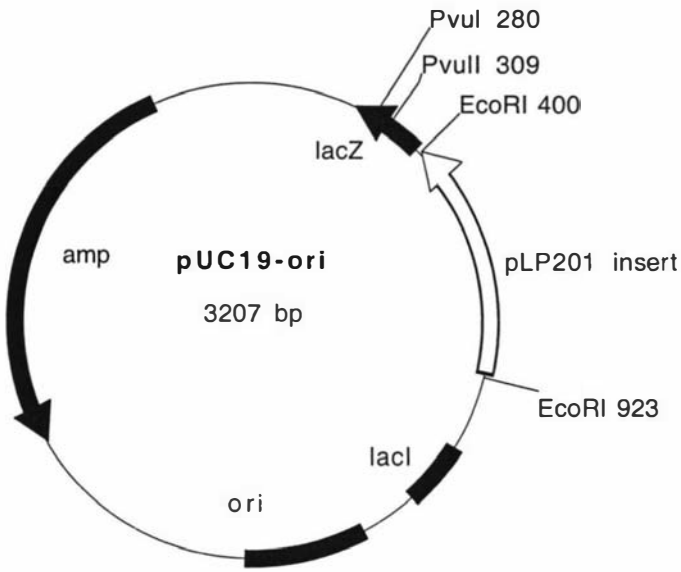
A1.22 Plasmid T7+207. 207 bp DNA sequence coding for the P_E1 transcript from c2 and T7 promoter (194 bp) cloned into pUC19.



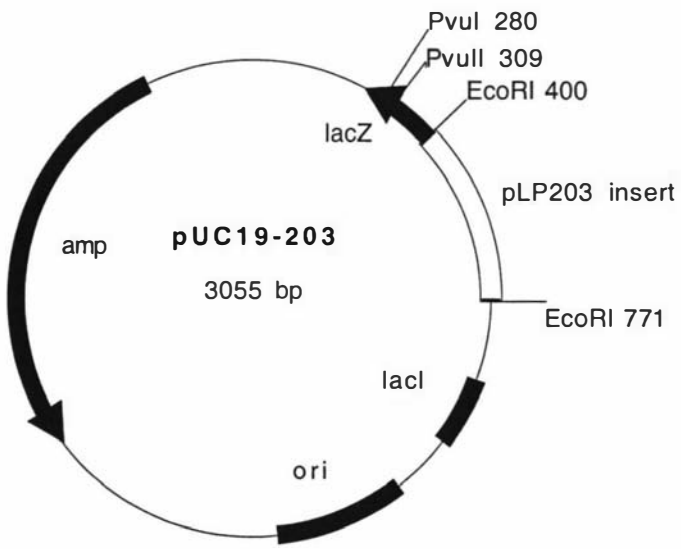
A1.23 Plasmid T7+307. 307 bp DNA sequence coding for the P_E1 transcript from c2 and T7 promoter (194 bp) cloned into pUC19.



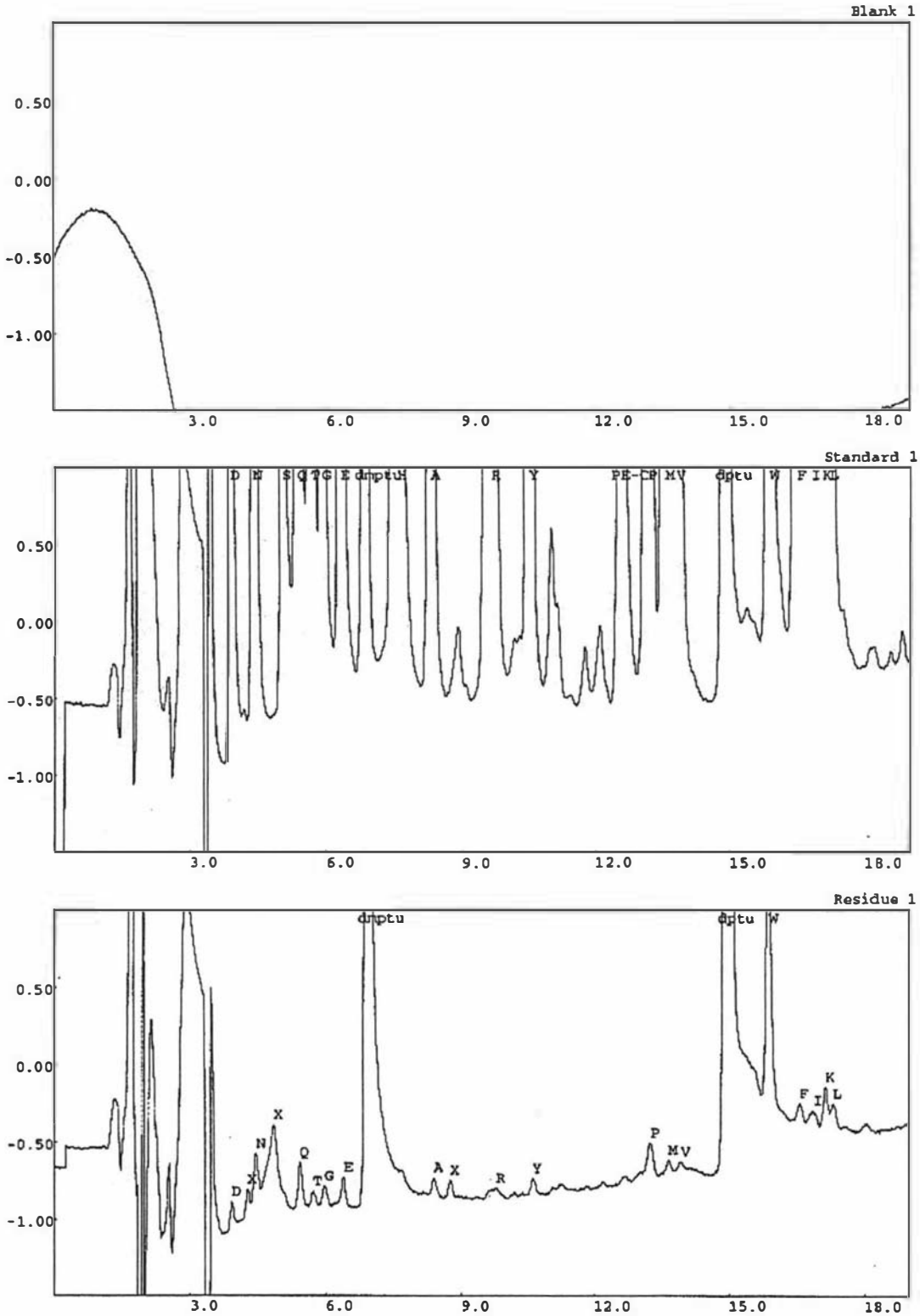
A1.24 Plasmid pUC19-*ori*. 521 bp c2 *ori* fragment from pLP201 cloned into pUC19.

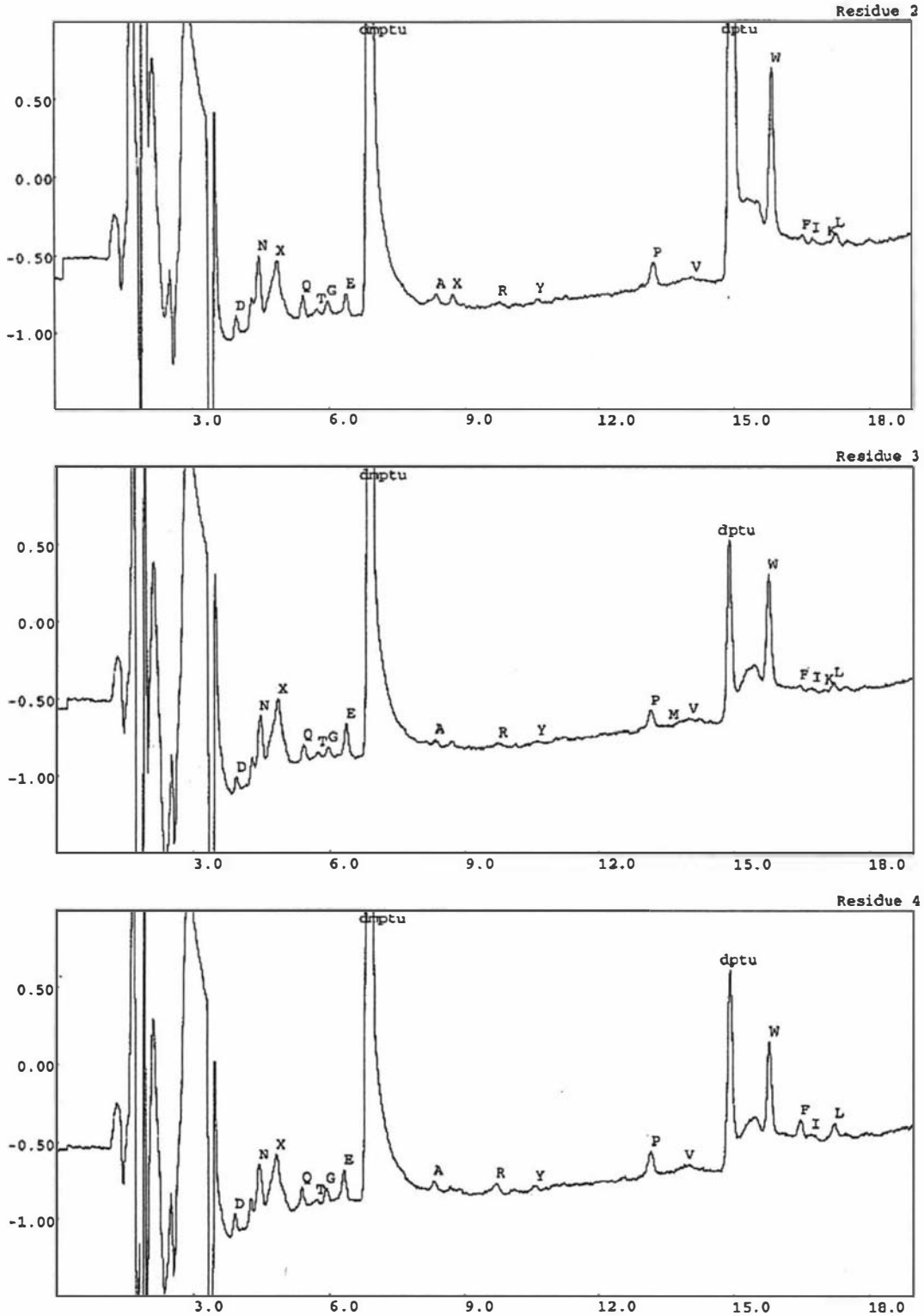


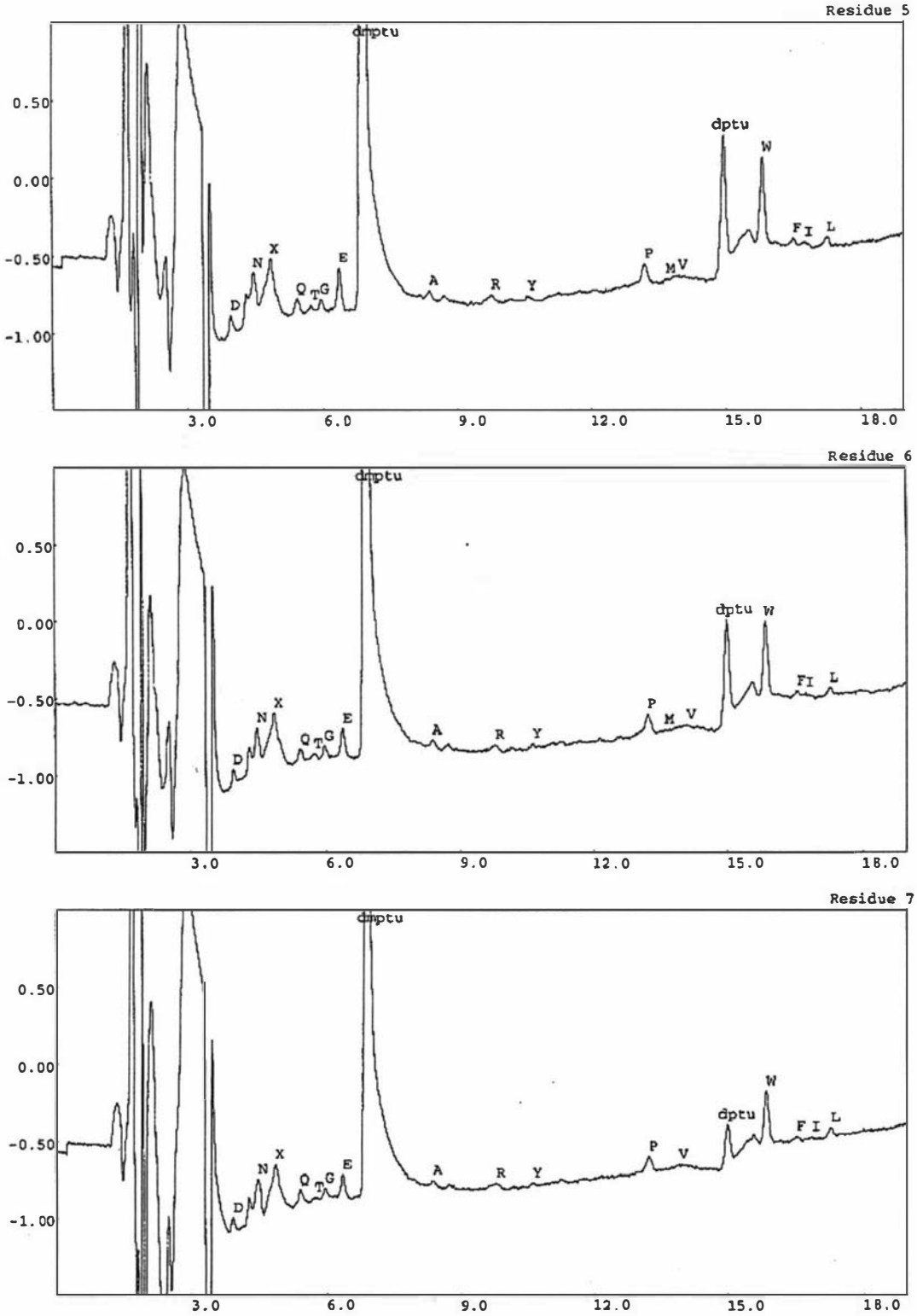
A1.25 Plasmid pUC19-203. 370 bp c2 *ori* fragment from pLP203 cloned into pUC19.

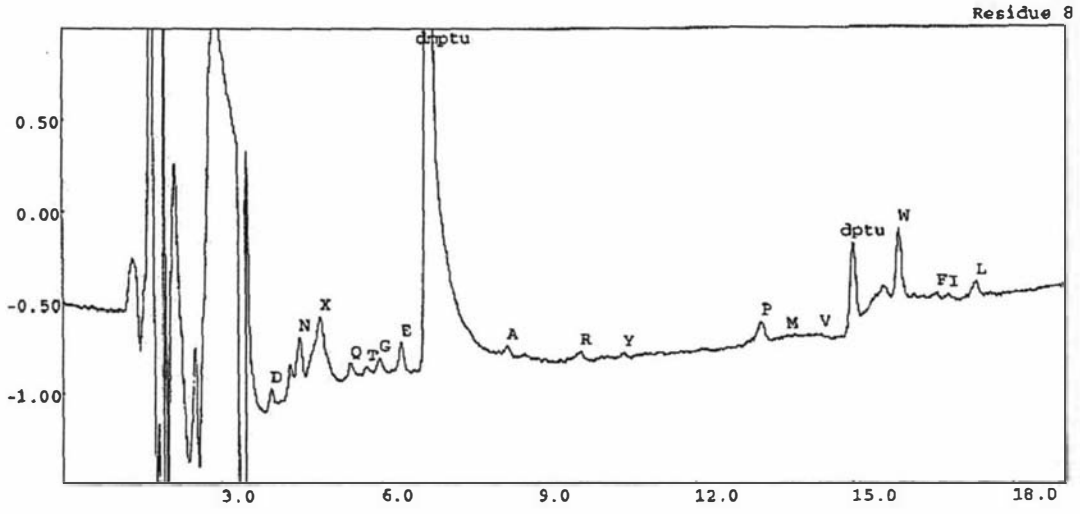


Appendix 2. Chromatographic data for cycles 1-8 of the Edman degradation of the 44 kDa protein binding to the 307 probe.




Residue 2
dmptu
dptu
W
0.50
0.00
-0.50
-1.00
N
X
D
Q
T
G
E
A
X
R
Y
P
V
F
I
K
L
3.0
6.0
9.0
12.0
15.0
18.0
Residue 3
dmptu
dptu
W
0.50
0.00
-0.50
-1.00
N
X
D
Q
T
G
E
A
R
Y
P
M
V
F
I
K
L
3.0
6.0
9.0
12.0
15.0
18.0
Residue 4
dmptu
dptu
W
0.50
0.00
-0.50
-1.00
N
X
D
Q
T
G
E
A
R
Y
P
V
F
I
L
3.0
6.0
9.0
12.0
15.0
18.0


Residue 5
dmptu
dptu
W
FI
L
P
MV
A
R
Y
N
X
E
Q
T
G
D
0.50
0.00
-0.50
-1.00
3.0
6.0
9.0
12.0
15.0
18.0
Residue 6
dmptu
dptu
W
FI
L
P
MV
A
R
Y
N
X
E
Q
T
G
D
0.50
0.00
-0.50
-1.00
3.0
6.0
9.0
12.0
15.0
18.0
Residue 7
dmptu
dptu
W
FI
L
P
V
A
R
Y
N
X
E
Q
T
G
D
0.50
0.00
-0.50
-1.00
3.0
6.0
9.0
12.0
15.0
18.0



The x-axis displays retention time [min], the y-axis absorbance units.

The N-terminal amino acid sequence determined for the 44 kDa protein was MNEFETLN. The amino acid sequence of the protein was obtained by overlaying the chromatograms obtained by Edman degradation for every cycle and comparing the peaks with the peaks obtained from the last cycle. An increased peak in a cycle reveals the amino acid determined.

Appendix 3. Growth curve analysis using the ANOVA procedure of the SAS system.

Displayed is the mean OD_{600} measurement for each growth curve after three independent experiments at every time point. Also displayed are the mean growth rates for every strain. To visualize if there was a difference in the growth rates during exponential growth the growth rates were grouped into A, B or AB. The same letter indicates that the means were not significantly different.

Mean OD_{600} measurement for strain MG1363

time after inoculation [hours]	OD_{600} mean					
	no plasmid	pTRKL2	pLP201	pLP203	pLP205	pLP206
0	0.050667	0.054	0.052	0.049	0.059	0.047
0.5	0.076333	0.077	0.074	0.072	0.088	0.076
1	0.09933	0.0985	0.104	0.106	0.124	0.101
1.5	0.157	0.14	0.137	0.155	0.197	0.128
2	0.254	0.215	0.186	0.205	0.306	0.188
2.5	0.395	0.322	0.277	0.31	0.449	0.243
3	0.57433	0.47	0.38	0.416	0.617	0.327
3.5	0.7847	0.628	0.466	0.505	0.815	0.448
4	1.02	0.836	0.629	0.677	0.838	0.676
4.5	1.181	0.974	0.778	0.828	1.141	0.822
5	1.249	1.137	0.996	1.02	1.239	0.921
5.5	1.29167	1.195	1.119	1.13	1.268	1.212
6	1.288	1.2075	1.174	1.173	1.298	1.252
6.5	1.232	1.235	1.219	1.201	1.257	1.37
7	1.236	1.24	1.172	1.163	1.259	1.35
24	1.275	1.23	1.177	1.185	1.226	1.27

Grouping for strain MG1363

	Growth rate	Grouping
no plasmid	0.33	A
pTRKL2	0.25	A
pLP201	0.26	A
pLP203	0.25	A
pLP205	0.26	A
pLP206	0.27	A

Mean OD_{600} measurement for strain NZ9000

time after inoculation [hours]	OD_{600} mean			
	no plasmid	pTRKL2	pLP212 +nisin	pLP212 -nisin
0	0.054	0.0507	0.0495	0.049
0.5	0.0835	0.072	0.0715	0.068
1	0.122	0.0937	0.099	0.094
1.5	0.187	0.1303	0.136	0.135
2	0.2935	0.1963	0.1855	0.174
2.5	0.4395	0.2847	0.289	0.295
3	0.619	0.4073	0.436	0.376
3.5	0.842	0.56	0.5435	0.495
4	1.0555	0.761	0.7355	0.609
4.5	1.205	0.961	0.8846	0.74
5	1.284	1.0957	1.0825	0.881
5.5	1.306	1.1823	1.209	1.12
6	1.2183	1.328	1.407	1.255
6.5	1.3	1.227	1.42	1.365
7	1.29	1.226	1.4	1.316
24	1.25	1.21	1.29	1.3

Grouping for strain NZ9000

	Growth rate	Grouping
no plasmid	0.3	A
pTRKL2	0.3	A
pLP212 +nisin	0.3	A
pLP212 –nisin	0.22	B

Mean OD_{600} measurement for strain NZ9800

time after inoculation	OD_{600} mean			
[hours]	no plasmid	pTRKL2	pLP212 +nisin	pLP212 -nisin
0	0.052667	0.055	0.055	0.05
0.5	0.076	0.0737	0.0685	0.068
1	0.107	0.0983	0.103	0.1105
1.5	0.16967	0.1407	0.1675	0.163
2	0.25467	0.1927	0.246	0.2545
2.5	0.37233	0.2667	0.39	0.367
3	0.53433	0.38	0.5475	0.5145
3.5	0.73	0.5123	0.76	0.7235
4	0.95567	0.6667	0.8805	0.9345
4.5	1.13567	0.862	1.0885	1.1405
5	1.25	1.0063	1.2815	1.2355
5.5	1.307	1.125	1.3245	1.291
6	1.307	1.223	1.3605	1.2995
6.5	1.318	1.236	1.35	1.3
7	1.323	1.238	1.365	1.32
24	1.326	1.246	1.29	1.26

Grouping for strain NZ9800

	Growth rate	Grouping
no plasmid	0.3	A
pTRKL2	0.27	A
pLP212 +nisin	0.3	A
pLP212 –nisin	0.3	A

References

Ackermann, H.-W. (1999). Tailed bacteriophages: the order *Caudovirales*. *Adv. Virus Res.* 51: 135-201.

Ackermann, H.-W. (2001). Frequency of morphological phage descriptions. *Arch. Virol.* 146: 843-857.

Agaisse, H. and Lereclus, D. (1996). STAB-SD: a Shine-Dalgarno sequence in the 5' untranslated region is a determinant of mRNA stability. *Mol. Microbiol.* 20: 633-643.

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.

Anba, J., Bidnenko, E., Hillier, A., Ehrlich, D. and Chopin, M. C. (1995). Characterization of the lactococcal *abiD1* gene coding for phage abortive infection. *J. Bacteriol.* 177: 3818-3823.

Andersen, J., Forst, S. A., Zhao, K., Inouye, M. and Delihas, N. (1989). The function of *micF* RNA. *micF* RNA is a major factor in the thermal regulation of OmpF protein in *Escherichia coli*. *J. Biol. Chem.* 264: 17961-17970.

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

Arendt, E. K., Daly, C., Fitzgerald, G. F. and van de Guchte, M. (1994). Molecular characterization of lactococcal bacteriophage Tuc2009 and identification and analysis of genes encoding lysin, a putative holin, and two structural proteins. *Appl. Environ. Microbiol.* 60: 1875-1883.

Baker, T. A. and Kornberg, A. (1988). Transcriptional activation of initiation of replication from the *E. coli* chromosomal origin: an RNA-DNA hybrid near *oriC*. *Cell* 55: 113-123.

Baker, T. A. and Bell, S. P. (1998). Polymerases and the replisome: machines within machines. *Cell* 92: 295-305.

Batt, C. A., Erlandson, K. and Bsat, N. (1995). Design and implementation of a strategy to reduce bacteriophage infection of dairy starter cultures. *Int. Dairy. J.* 5: 949-962.

Belanger, K. G. and Kreuzer, K. N. (1998). Bacteriophage T4 initiates bidirectional DNA replication through a two-step process. *Mol. Cell* 2: 693-701.

Belasco, J. G., Beatty, J. T., Adams, C. W., von Gabain, A. and Cohen, S. N. (1985). Differential expression of photosynthesis genes in *R. capsulata* results from segmental differences in stability within the polycistronic *rxcA* transcript. *Cell* 40: 171-181.

Belasco, J. G. and Higgins, C. F. (1988). Mechanisms of mRNA decay in bacteria: a perspective. *Gene* 72: 15-23.

Benkovic, S. J., Valentine, A. M. and Salinas, F. (2001). Replisome-mediated DNA replication. *Annu. Rev. Biochem.* 70: 181-208.

Beresford, T. P. J., Ward, L. J. H. and Jarvis, A. W. (1993). Temporally regulated transcriptional expression of the genomes of lactococcal bacteriophages c2 and sk1. *Appl. Environ. Microbiol.* 59: 3708-3712.

Bergh, O., Børsheim, K. Y., Bratbak, G. and Heldal, M. (1989). High abundance of viruses found in aquatic environments. *Nature* 340: 467-468.

Bernstein, J. A., Khodursky, A. B., Lin, P.-H., Lin-Chao, S. and Cohen, S. N. (2002). Global analysis of mRNA decay and abundance in *Escherichia coli* at single-gene resolution using two-color fluorescent DNA microarrays. *Proc. Natl. Acad. Sci. USA* 99: 9697-9702.

Bidnenko, E., Ehrlich, D. and Chopin, M. C. (1995). Phage operon involved in sensitivity to the *Lactococcus lactis* abortive infection mechanism AbiD1. *J. Bacteriol.* 177: 3824-3829.

Bidnenko, E., Ehrlich, S. D. and Chopin, M. C. (1998). *Lactococcus lactis* phage operon coding for an endonuclease homologous to RuvC. *Mol. Microbiol.* 28: 823-834.

Binnie, U., Wong, K., McAteer, S. and Masters, M. (1999). Absence of RNase III alters the pathway by which RNAI, the antisense inhibitor of ColE1 replication, decays. *Microbiology* 145: 3089-3100.

Birge, E. A. (2000). *Bacterial and Bacteriophage Genetics.* 4th edition, Springer-Verlag New York.

Biswas, B., Adhya, S., Washart, P., Paul, B., Trostel, A. N., Powell, B., Carlton, R. and Merril, C. R. (2002). Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *Enterococcus faecium*. *Infect. Immun.* 70: 204-210.

Blatny, J. M., Risøen, P. A., Lillehaug, D., Lunde, M. and Nes, I. (2001). Analysis of a regulator involved in the genetic switch between lysis and lysogeny of the temperate *Lactococcus lactis* phage ϕLC3. *Mol. Genet. Genomics* 265: 189-197.

Blumenthal, T. and Carmichael, G. G. (1979). RNA replication: function and structure of Qβ-replicase. *Annu. Rev. Biochem.* 48: 525-548.

Bolotin, A., Mauger, S., Malarme, K., Ehrlich, S. D. and Sorokin, A. (1999). Low-redundancy sequencing of the entire *Lactococcus lactis* IL1403 genome. *Antonie van Leeuwenhoek* 76: 27-76.

Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarme, K., Weissenbach, J., Ehrlich, S. D. and Sorokin, A. (2001). The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res.* 11: 731-753.

Botstein, D., Lew, K. K., Jarvik, V. and Swanson, C. A. (1975). Role of antirepressor in the bipartite control of repression and immunity by bacteriophage P22. *J. Mol. Biol.* 91: 439-462.

Bouchard, J. D., and Moineau, S. (2000). Homologous recombination between a lactococcal bacteriophage and the chromosome of its host strain. *Virology* 270: 65-75.

Bouchard, J. D., Dion, E. and Moineau, S. (2000). Characterization of a novel phage-resistance mechanism in *Lactococcus lactis*. *J. Dairy Sci.* 83 (Suppl. 1): 132.

Bouchard, J. D., Dion, E., Bissonnette, F. and Moineau, S. (2002). Characterization of the two-component abortive phage infection mechanism AbiT from *Lactococcus lactis*. *J. Bacteriol.* 184: 6325-6332.

Boucher, I., Émond, É., Dion, É., Montpetit, D. and Moineau, S. (2000). Microbiological and molecular impacts of AbiK on the lytic cycle of *Lactococcus lactis* phages of the 936 and P335 species. *Microbiology* 146: 445-453.

Boucher, I., Émond, É., Parrot, M. and Moineau, S. (2001). DNA sequence analysis of three *Lactococcus lactis* plasmids encoding phage resistance mechanisms. *J. Dairy Sci.* 84: 1610-1620.

Bourdeau, V., Ferbeyre, G., Pageau, M., Paquin, B. and Cedergren, R. (1999). The distribution of RNA motifs in natural sequences. *Nucleic Acids Res.* 27: 4457-4467.

Boyce, J. D., Davidson, B. E. and Hillier, A. J. (1995). Sequence analysis of the *Lactococcus lactis* temperate bacteriophage BK5-T and demonstration that the phage DNA has cohesive ends. *Appl. Environ. Microbiol.* 61: 4089-4098.

Braasch, D. A. and Corey, D. R. (2002). Novel antisense and peptide nucleic strategies for controlling gene expression. *Biochemistry* 41: 4503-4510.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.

Brakmann, S. and Grzeszik, S. (2001). An error-prone T7 RNA polymerase mutant generated by direct evolution. *Chembiochem.* 2: 212-219.

Bramhill, D. and Kornberg, A. (1988). A model for initiation at origins of DNA replication. *Cell* 54: 915-918.

Breüner, A., Brøndsted, L. and Hammer, K. (2001). Resolvase-like recombination performed by the TP901-1 integrase. *Microbiology* 147: 2051-2063.

Brøndsted, L., Østergaard, S., Pedersen, M., Hammer, K. and Vogensen, F. K. (2001). Analysis of the complete DNA sequence of the temperate bacteriophage TP901-1: evolution, structure, and genome organization of lactococcal bacteriophages. *Virology* 283: 93-109.

Brown, D. and Gold, L. (1995). Selection and characterization of RNAs replicated by Qβ replicase. *Biochemistry.* 34: 14775-14782.

Bruand, C., Le Chatelier, E., Ehrlich, S. D. and Jannière, L. (1993). A fourth class of theta-replicating plasmids: the pAMβ1 family from Gram-positive bacteria. *Proc. Natl. Acad. Sci. USA* 90: 11668-11672.

Bruand, C. and Ehrlich, S. D. (1998). Transcription-driven DNA replication of plasmid pAMβ1 in *Bacillus subtilis. Mol. Microbiol.* 30: 135-145.

Brüssow, H. (2001). Phages of dairy bacteria. *Annu. Rev. Microbiol.* 55: 283-303.

Brüssow, H. and Desiere, F. (2001). Comparative phage genomics and the evolution of *Siphoviridae*: insights from dairy phages. *Mol. Microbiol.* 39: 213-222.

Brüssow, H., and Hendrix, R. W. (2002). Phage genomics: small is beautiful. *Cell* 108**:** 13-16.

Bryan, E. M., Bae, T., Kleerebezem, M. and Dunny, G. M. (2000). Improved vectors for nisin-controlled expression in Gram-positive bacteria. *Plasmid* 44: 183-190.

Bycroft, M., Hubbard, T. J. P., Proctor, M., Freund, S. M. V. and Murzin, A. G. (1997). The solution of the S1 RNA binding domain: a member of an ancient nucleic acid-binding fold. *Cell* 88: 235-242.

Callanan, M. J., O'Toole, P. W., Lubbers, M. W. and Polzin, K. M. (2001). Examination of lactococcal bacteriophage c2 DNA replication using two-dimensional agarose gel electrophoresis. *Gene* 278: 101-106.

Campbell, A. (1994). Comparative molecular biology of lambdoid phages. *Annu. Rev. Microbiol* 48: 193-222.

Carles-Kinch, K. and Kreuzer, K. N. (1997). RNA-DNA hybrid formation at a bacteriophage T4 replication origin. *J. Mol. Biol.* 266: 915-926.

Carpousis, A. J., Vanyo, N. F. and Raznal, L. C. (1999). mRNA degradation. A tale of poly(A) and multiprotein machines. *Trends Genet.* 15: 24-28.

Carpousis, A. J. (2002). The *Escherichia coli* RNA degradosome: structure, function and relationship to other ribonucleolytic multienzyme complexes. *Biochem. Soc. Trans.* 30:150-155.

Carrier, T. A. and Keasling, J. D. (1997). Engineering mRNA stability in *E. coli* by the addition of synthetic hairpins using a 5' cassette system. *Biotechnol. Bioeng.* 55: 577-580.

Chandrapati, S. and O'Sullivan, D. J. (1999). Nisin independent induction of the *nisA* promoter in *Lactococcus lactis* during growth in lactose or galactose. *FEMS Microbiol. Lett.* 170: 191-198.

Chandry, P. S., Moore, S. C., Davidson, B. E. and Hillier, A. J. (1994). Analysis of the *cos* region of the *Lactococcus lactis* bacteriophage sk1. *Gene* 138: 123-126.

Chandry, P. S., Moore, S. C., Boyce, J. D., Davidson, B. E. and Hillier, A. J. (1997). Analysis of the DNA sequence, gene expression, origin of replication and modular structure of the *Lactococcus lactis* lytic bacteriophage sk1. *Mol. Microbiol.* 26: 49-64.

Chang, D. D. and Clayton, D. A. (1985). Priming of human mitochondrial DNA replication occurs at the light-strand promoter. *Proc. Natl. Acad. Sci. USA* 82: 351-355.

Chattoraj, D. K. (2000). Control of plasmid DNA replication by iterons: no longer paradoxical. *Mol. Microbiol.* 37: 467-476.

Chiaruttini, C. and Milet, M. (1993). Gene organization, primary structure and RNA processing analysis of a ribosomal RNA operon in *Lactococcus lactis*. *J. Mol. Biol.* 230: 57-76.

Chopin, M.-C., Chopin, A. and Roux, C. (1976). Definition of bacteriophage groups according to their lytic action on mesophilic lactic streptococci. *Appl. Environ. Microbiol.* 32: 741-746.

Chopin, A., Bolotin, A., Sorokin, A., Ehrlich, S. D. and Chopin, M.-C. (2001). Analysis of six prophages in *Lactococcus lactis* IL1403: different genetic structure of temperate and virulent phage populations. *Nucleic Acids Res.* 29: 644-651.

Christensson, C., Pillidge, C. J., Ward, L. J. H. and O'Toole, P. W. (2001). Nucleotide sequence and characterization of the cell envelope proteinase plasmid in *Lactococcus lactis* subsp. *cremoris* HP. *J. Appl. Microbiol.* 91: 334-343.

Chung, C. T., Niemela, S. L. and Miller, R. H. (1989). One-step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. USA*: 2172-2175.

Chung, D. K., Kim, J. H. and Batt, C. A. (1991). Cloning and nucleotide sequence of the major capsid protein from *Lactococcus lactis* ssp. *cremoris* bacteriophage F4-1. *Gene* 101: 121-125.

Chung, D. K., Chung, S. K. and Batt, C. A. (1992). Antisense RNA directed against the major capsid protein of *Lactococcus lactis* subsp. *cremoris* bacteriophage 4-1 confers partial resistance to the host. *Appl. Microbiol. Technol.* 37: 79-83.

Cluzel, P.-J., Chopin, A., Ehrlich, S. D. and Chopin, M.-C. (1991). Phage abortive infection mechanism from *Lactococcus lactis* subsp. *lactis*, expression of which is mediated by an Iso-IS*S1* element. *Appl. Environ. Microbiol.* 57: 3547-3551.

Coffey, A. G., Fitzgerald, G. F. and Daly, C. (1991). Cloning and characterization of the determinant for abortive infection of bacteriophage from lactococcal plasmid pCI829. *J. Gen. Microbiol.* 137: 1355-1362.

Coffey, A. and Ross, R. P. (2002). Bacteriophage-resistance systems in dairy starter strains: molecular analysis to application. *Antonie van Leeuwenhoek* 82: 303-321.

Cook, P. R. (1999). The organization of replication and transcription. *Science* 284: 1790-1795.

Côté, J. and Ruiz-Carrillo, A. (1993). Primers for mitochondrial DNA replication generated by endonuclease G. *Science* 261: 765-769.

Coulombe, B. and Burton, Z. (1999). DNA bending and wrapping around RNA polymerase: a "revolutionary" model describing transcriptional mechanisms. *Microbiol. Mol. Biol. Rev.* 63: 457-478.

Crick, F. H. C., Barnett, L., Brenner, S. and Watts-Tobin, R. J. (1961). General nature of the genetic code for proteins. *Nature* 192: 1227-1232.

Crooke, E. (1995). DNA synthesis initiated at *oriC*: *in vitro* replication reactions. *Methods Enzymol.* 262: 500-506.

Crutz-Le Coq, A.-M., Cesselin, B., Commissaire, J. and Anba, J. (2002). Sequence analysis of the lactococcal bacteriophage bIL170: insights into structural proteins and HNH endonucleases in dairy phages. *Microbiology* 148: 985-1001.

Dai, G., Su, P., Allison, G. E., Geller, B. L., Zhu, P., Kim, W. S. and Dunn, N. W. (2001). Molecular characterization of a new abortive infection system (AbiU) from *Lactococcus lactis* LL51-1. *Appl. Environ. Microbiol.* 67: 5225-5232.

Daly, C., Fitzgerald, G. F. and Davis, R. (1996). Biotechnology of lactic acid bacteria with special reference to bacteriophage resistance. *Antonie van Leeuwenhoek* 70: 99-110.

Dasgupta, S., Masukata, H. and Tomizawa, J. (1987). Multiple mechanisms for initiation of ColE1 DNA replication: DNA synthesis in the presence and absence of ribonuclease H. *Cell* 51: 1113-1122.

Delves-Broughton, J. (1998). Nisin. *Bulletin of the International Dairy Federation* 329: 9-12.

Deng, Y. M., Harvey, M. L., Liu, C. Q. and Dunn, N. W. (1997). A novel plasmid-encoded phage abortive infection system from *Lactococcus lactis* biovar. *diacetylactis. FEMS Microbiol. Lett.* 146: 149-154.

Deng, Y. M., Liu, C. Q. and Dunn, N. W. (1999). Genetic organization and functional analysis of a novel phage abortive infection system, AbiL, from *Lactococcus lactis. J. Biotechnol.* 67: 135-149.

DePamphilis, M. L. (1988). Transcriptional elements as components of eukaryotic origins of DNA replication. *Cell* 52: 635-638.

de Rijk, P. and de Wachter, R. (1997). RnaViz, a program for the visualization of RNA secondary structure. *Nucleic Acids Res.* 25: 4679-4684.

de Ruyter, P. G., Kuipers, O. P., Beerthuyzen, M. M., van Alen-Boerrigter, I. and de Vos, W. M. (1996a). Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis. J. Bacteriol.* 178: 3434-3439.

de Ruyter, P. G. G. A., Kuipers, O. P. and de Vos, W. M. (1996b). Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl. Environ. Microbiol.* 62: 3662-3667.

Desiere, F., McShan, W. M., van Sinderen, D., Ferretti, J. J. and Brüssow, H. (2001). Comparative genomics reveals close genetic relationships between phages from dairy bacteria and pathogenic Streptococci: evolutionary implications for prophage-host interactions. *Virology* 288: 325-341.

Desiere, F., Lucchini, S., Canchaya, C., Ventura, M. and Brüssow, H. (2002). Comparative genomics of phages and prophages in lactic acid bacteria. *Antonie van Leeuwenhoek* 82: 73-91.

Dinsmore, P. K. and Klaenhammer, T. R. (1994). Phenotypic consequences of altering the copy number of *abiA*, a gene responsible for aborting bacteriophage infections in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 60: 1129-1136.

Dinsmore, P. K. and Klaenhammer, T. R. (1997). Molecular characterization of a genomic region in a *Lactococcus* bacteriophage that is involved in its sensitivity to the phage defense mechanism AbiA. *J. Bacteriol.* 179: 2949-2957.

Dinsmore, P. K., O'Sullivan, D. J. and Klaenhammer, T. R. (1998). A leucine repeat motif in AbiA is required for resistance of *Lactococcus lactis* to phages representing three species. *Gene* 212: 5-11.

Djordjevic, G. M. and Klaenhammer, T. R. (1997a). Bacteriophage-triggered defense systems: phage adaptation and design improvements. *Appl. Environ. Microbiol.* 63: 4370-4376.

Djordjevic, G. M. and Klaenhammer, T. R. (1997b). Genes and gene expression in *Lactococcus* bacteriophages. *Int. Dairy J.* 7: 489-508.

Djordjevic, G. M., O'Sullivan, D. J., Walker, S. A., Conkling, M. A. and Klaenhammer, T. R. (1997). A triggered-suicide system designed as a defense against bacteriophages. *J. Bacteriol.* 179: 6741-6748.

Dodd, H. M., Horn, N. and Gasson, M. J. (1990). Analysis of the genetic determinant for production of the peptide antibiotic nisin. *J. Gen. Microbiol.* 136: 555-566.

Drider, D., Santos, J. M., Arraiano, C. M. and López, P. (1998). RNA processing is involved in the post-transcriptional control of the *citQRP* operon from *Lactococcus lactis* biovar *diacetylactis*. *Mol. Gen. Genet.* 258: 9-15.

Drider, D., Bolotine, A., Renault, P. and Prévost, H. (2002). Functional study of *Lactococcus lactis* RNase III in *Escherichia coli*. *Plasmid* 47: 246-250.

Durmaz, E., Higgins, D. L. and Klaenhammer, T. R. (1992). Molecular characterization of a second abortive phage resistance gene present in *Lactococcus lactis* subsp. *lactis* ME2. *J. Bacteriol.* 174: 7463-7469.

Durmaz, E. and Klaenhammer, T. R. (2000). Genetic analysis of chromosomal regions of *Lactococcus lactis* acquired by recombinant lytic phages. *Appl. Environ. Microbiol.* 66: 895-903.

Duwat, P., Ehrlich, S. D. and Gruss, A. (1999). Effects of metabolic flux on stress response pathways in *Lactococcus lactis*. *Mol. Microbiol.* 31: 845-858.

Eichenbaum, Z., Federle, M. J., Marra, D., de Vos, W. M., Kuipers, O. P., Kleerebezem, M. and Scott, J. R. (1998). Use of the lactococcal *nisA* promoter to regulate gene expression in gram-positive bacteria: comparison of induction level and promoter strength. *Appl. Environ. Microbiol.* 64: 2763-2769.

Émond, E., Holler, B. J., Boucher, I., Vandenbergh, P. A., Vedamuthu, E. R., Kondo, J. K. and Moineau, S. (1997). Phenotypic and genetic characterization of the bacteriophage abortive infection mechanism AbiK from *Lactococcus lactis*. *Appl. Environ. Microbiol.* 63: 1274-1283.

Émond, E., Dion, E., Walker, S. A., Vedamuthu, E. R., Kondo, J. K. and Moineau, S. (1998). AbiQ, an abortive infection mechanism from *Lactococcus lactis*. *Appl. Environ. Microbiol.* 64: 4748-4756.

Fang, L., Davey, M. J. and O'Donnell, M. (1999). Replisome assembly at *oriC*, the replication origin of *E. coli*, reveals an explanation for initiation sites outside an origin. *Mol. Cell* 4: 541-553.

Farkasovská, J., Godány, A., Bukovská, G. and Timko, J. (2001). An origin of DNA replication from streptomycete phage ϕU1. *Folia Microbiol.* 46: 483-487.

Feng, Y., Huang, H., Liao, J. and Cohen, S. N. (2001). *Escherichia coli* poly(A)-binding proteins that interact with components of degradosomes or impede RNA decay mediated by polynucleotide phosphorylase and RNase E. *J. Biol. Chem.* 276: 31651-31656.

Fernández, A., Guo, H. S., Saenz, P., Simon-Buela, L., Gomez de Cedron, M. and Garcia, J. A. (1997). The motif V of plum pox potyvirus CI RNA helicase is involved in NTP hydrolysis and is essential for virus RNA replication. *Nucleic Acids Res.* 25: 4474-4480.

Foley, S., Lucchini, S., Zwahlen, M. C. and Brüssow, H. (1998). A short noncoding viral DNA element showing characteristics of a replication origin confers bacteriophage resistance to *Streptococcus thermophilus*. *Virology* 250: 377-387.

Forde, A., Daly, C. and Fitzgerald, G. F. (1999). Identification of four phage resistance plasmids from *Lactococcus lactis* subsp. *cremoris* HO2. *Appl. Environ. Microbiol.* 65: 1540-1547.

Forde, A. and Fitzgerald, G. F. (1999). Bacteriophage defence systems in lactic acid bacteria. *Antonie van Leeuwenhoek* 76: 89-113.

Frey, J. and Krisch, H. M. (1985). Ω mutagenesis in gram-negative bacteria: a selectable interposon which is strongly polar in a wide range of bacterial species. *Gene* 36: 143-150.

Frick, D. N. and Richardson, C. C. (2001). DNA primases. *Annu. Rev. Biochem.* 70: 39-80.

Gabrielsen, O. S., Hornes, E., Korsnes, L., Ruet, A. and Øyen, T. B. (1989). Magnetic DNA affinity purification of yeast transcription factor τ – a new purification principle for the ultrarapid isolation of near homogeneous factor. *Nucleic Acids Res.* 17: 6253-6267.

Garbutt, K. C., Kraus, J. and Geller, B. L. (1997). Bacteriophage resistance in *Lactococcus lactis* engineered by replacement of a gene for a bacteriophage receptor. *J. Dairy Sci.* 80: 1512-1519.

Garvey, P., van Sinderen, D., Twomey, D. P., Hill, C. and Fitzgerald, G. F. (1995a). Molecular genetics of bacteriophage and natural phage defence systems in the genus *Lactococcus*. *Int. Dairy J.* 5: 905-947.

Garvey, P., Fitzgerald, G. F. and Hill, C. (1995b). Cloning and DNA sequence analysis of two abortive infection phage resistance determinants from the lactococcal plasmid pNP40. *Appl. Environ. Microbiol.* 61: 4321-4328.

Garvey, P., Hill, C., and Fitzgerald, G. F. (1996). The lactococcal plasmid pNP40 encodes a third bacteriophage resistance mechanism, one which affects phage DNA penetration. *Appl. Environ. Microbiol.* 62: 676-679.

Gasson, M. J. (1983). Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.* 154: 1-9.

Gasson, J. J. and de Vos, W. M. (1994). Genetics and biotechnology of lactic acid bacteria 1/e. *Blackie Academic & Professional, Glasgow, UK.*

Gasson, J.M. (1996). Lytic systems in lactic acid bacteria and their bacteriophages. *Antonie van Leeuwenhoek* 70: 147-159.

Geller, B. L., Ivey, R. G., Trempy, J. E. and Hettinger-Smith, B. (1993). Cloning of a chromosomal gene required for phage infection of *Lactococcus lactis* subsp. *lactis* C2. *J. Bacteriol.* 175: 5510-5519.

Georgopoulos, C. P., Hendrix, R. W., Casjens, S. R. and Kaiser, A. D. (1973). Host participation in bacteriophage lambda head assembly. *J. Mol. Biol.* 76: 45-60.

Giedroc, D. P., Theimer, C. A. and Nixon, P. L. (2000). Structure, stability and function of RNA pseudoknots involved in stimulating ribosomal frameshifting. *J. Mol. Biol.* 298: 167-185.

Giorginis, S. and Subramanian, A. R. (1980). The major ribosome binding site of *Escherichia coli* ribosomal protein S1 is located in its N-terminal segment. *J. Mol. Biol.* 141: 393-408.

Gordon, J. (1997). Investigation of an origin of replication of the lactococcal bacteriophage c2. Bachelor of Science (Honours) thesis in Microbiology at Massey University, Palmerston North, New Zealand

Goulian, M. and Kornberg, A. (1967). Enzymatic synthesis of DNA, XXIII. synthesis of circular replicative form of phage ϕX174 DNA. *Proc. Natl. Acad. Sci. USA* 58: 1723-1730.

Grohmann, E., Moscoso, M., Zechner, E. L., del Solar, G. and Espinosa, M. (1998). *In vivo* definition of the functional origin of leading strand replication on the lactococcal plasmid pFX2. *Mol. Gen. Genet.* 260: 38-47.

Guarneros, G. and Galindo, J. M. (1979). The regulation of integrative recombination by the *b2* region and the *c*II gene of bacteriophage λ. *Virology* 95: 119-126.

Gutiérrez-Escolano, A. L., Brito, U. Z., Del Angel, R. M. and Jiang, X. (2000). Interaction of cellular proteins with the 5' end of Norwalk virus genomic RNA. *J. Virol.* 74: 8558-8562.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166: 557-580.

Hase, T., Nakai, M. and Masamune, Y. (1989). Transcription of a region downstream from lambda *ori* is required for replication of plasmids derived from coliphage lambda. *Mol. Gen. Genet.* 216: 120-125.

Hassan, A. B. and Cook, P. R. (1994). Does transcription by RNA polymerase play a direct role in the initiation of replication? *J. Cell Sci.* 107: 1381-1387.

Heap, H. A. and Jarvis, A. W. (1980). A comparison of prolate and isometric-headed lactic streptococcal bacteriophages. *N. Z. J. Dairy Sci. Technol.* 15: 75-81.

Held, W. A., Mizushima, S. and Nomura, M. (1973). Reconstitution of *Escherichia coli* 30S ribosomal subunits from purified molecular components. *J. Biol. Chem.* 248: 5720-5730.

Henkin, T. M. (1996). Control of transcription termination in prokaryotes. *Annu. Rev. Genet.* 30: 35-57.

Hermann, T. and Patel, D. J. (1999). Stitching together RNA tertiary architectures. *J. Mol. Biol.* 294: 829-849.

Herrin, D. L. and Schmidt, G. W. (1988). Rapid, reversible staining of Northern blots prior to hybridization. *BioTechniques* 6: 196-197.

Hershey, A. D. and Chase, M. (1952). Independent function of viral protein and nucleic acid in growth of bacteriophage. *J. Gen. Physiol.* 36: 39-56.

Higgs, P. G. (2000). RNA secondary structure: physical and computational aspects. *Q. Rev. Biophys.* 33: 199-253.

Hill, C., Miller, L. A. and Klaenhammer, T. R. (1990a). Nucleotide sequence and distribution of the pTR2030 resistance determinant (*hsp*) which aborts bacteriophage infection in lactococci. *Appl. Environ. Microbiol.* 56: 2255-2258.

Hill, C., Miller, L. A. and Klaenhammer, T. R. (1990b). Cloning, expression, and sequence determination of a bacteriophage fragment encoding bacteriophage resistance in *Lactococcus lactis*. *J. Bacteriol.* 172: 6419-6426.

Hill, C., Miller, L. A. and Klaenhammer, T. R. (1991). *In vivo* genetic exchange of a functional domain from a type II A methylase between lactococcal plasmid pTR2030 and a virulent bacteriophage. *J. Bacteriol.* 173: 4363-4370.

Holo, H. and Nes, I. F. (1989). High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl. Environ. Microbiol.* 55: 3119-3123.

Holzapfel, W. H. and Wood, B. J. B. (1995). Lactic acid bacteria in contemporary perspective. *The genera of lactic acid bacteria*: 1-234. First edition, Blackie Academic and Professional, Glasgow, UK.

Horodniceanu, T., Bouanchaud, D. H., Bieth, G. and Chabbert, Y. A. (1976). R plasmids in *Streptococcus agalactiae* (group B). *Antimicrob. Agents Chemother.* 10: 795-801.

Itoh, T. and Tomizawa, J.-I. (1980). Formation of an RNA primer for initiation of replication of ColE1 DNA by ribonuclease H. *Proc. Natl. Acad. Sci. USA* 77: 2450-2454.

Jarvis, A. W. (1977). The serological differentiation of lactic streptococcal bacteriophage. *N. Z. J. Dairy Sci. Technol.* 12: 176-181.

Jarvis, A. W. (1984). Differentiation of lactic streptococcal phages into phage species by DNA-DNA homology. *Appl. Environ. Microbiol.* 47: 343-349.

Jarvis, A. W. and Klaenhammer, T. R. (1986). Bacteriophage resistance conferred on lactic streptococci by the conjugative plasmid pTR2030: Effects on small isometric-, large isometric-, and prolate-headed phages. *Appl. Environ. Microbiol.* 51: 1272-1277.

Jarvis, A. W. (1988). Conjugal transfer in lactic streptococci of plasmid-encoded insensitivity to prolate- and small isometric-headed bacteriophages. *Appl. Environ. Microbiol.* 54: 777-783.

Jarvis, A. W. (1989). Bacteriophages of lactic acid bacteria. *J. Dairy Sci.* 72: 3406-3428.

Jarvis, A. W., Fitzgerald, G. F., Mata, M., Mercenier, A., Neve, H., Powell, I. B., Ronda, C., Saxelin, M. and Teuber, M. (1991). Species and type phages of lactococcal bacteriophages. *Intervirology* 32: 2-9.

Jarvis, A. W. (1992). Analysis of phage resistance mechanisms encoded by lactococcal plasmid pAJ2074. *Can. J. Microbiol.* 39: 252-258.

Jarvis, A. W., Lubbers, M. W., Waterfield, N. R., Collins, L. J. and Polzin, K. M. (1995). Sequencing and analysis of the genome of lactococcal phage c2. *Int. Dairy J.* 5: 963-976.

Jensen, P. R. and Hammer, K. (1998). The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters. *Appl. Environ. Microbiol.* 64: 82-87.

Kain, K. C., Orlandi, P. A. and Lanar, D. E. (1991). Universal promoter for gene expression without cloning: expression-PCR. *BioTechniques* 10: 166-373.

Katayama, T. (2001). Feedback controls restrain the initiation of *Escherichia coli* chromosomal replication. *Mol. Microbiol.* 41: 9-17.

Kato, J.-I. and Katayama, T. (2001). Hda, a novel DnaA-related protein, regulates the replication cycle in *Escherichia coli. EMBO J.* 20: 4253-4262.

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

Khan, S. A. (2000). Plasmid rolling-circle replication: recent developments. *Mol. Microbiol.* 37: 477-484.

Kiewiet, R., Kok, J., Seegers, J. F. M. L., Venema, G. and Bron, S. (1993). The mode of replication is a major factor in segregational plasmid instability in *Lactococcus lactis. Appl. Environ. Microbiol.* 59: 358-364.

Kim, J. H., Kim, S. G., Chung, D. K., Bor, Y. C. and Batt, C. A. (1992). Use of antisense RNA to confer bacteriophage resistance in dairy starter cultures. *J. Ind. Microbiol.* 10: 71-78.

Klovins, J. and van Duin, J. (1999). A long-range pseudoknot in Qβ RNA is essential for replication. *J. Mol. Biol.* 294: 875-884.

Kondo, J. K., and Johansen, E. (2002). Product development strategies for foods in the era of molecular biotechnology. *Antonie van Leeuwenhoek.* 82: 291-302.

Konings, W. N., Kok, J., Kuipers, O. P. and Poolman, B. (2000). Lactic acid bacteria: the bugs of the new millennium. *Curr. Opin. Microbiol.* 3: 276-282.

Kornberg, A. and Baker, T. A. (1992). *DNA Replication.* 2nd Edition (Freeman, W. H., Ed.), New York.

Kowalski, D. and Sanford, J. P. (1982). Action of mung bean nuclease on supercoiled PM2 DNA. *J. Biol. Chem.* 257: 7820-7825.

Kraus, J. and Geller, B. L. (2001). Cloning of genomic DNA of *Lactococcus lactis* that restores phage sensitivity to an unusual bacteriophage sk1-resistant mutant. *Appl. Environ. Microbiol.* 67: 791-798.

Kuipers, O. P., Beerthuyzen, M. M., Siezen, R. J. and de Vos, W. M. (1993). Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*: requirement of expression of the *nisA* and *nisI* genes for development of immunity. *Eur. J. Biochem.* 216: 281-291.

Kuipers, O. P., Beerthuyzen, M. M., de Ruyter, P. G. G. A., Luesink, E. J. and de Vos, W. M. (1995). Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. *J. Biol. Chem.* 270: 27299-27304.

Kuipers, O. P., de Ruyter, P. G. G. A., Kleerebezem, M. and de Vos, W. M. (1998). Quorum sensing-controlled gene expression in lactic acid bacteria. *J. Biotechnol.* 64: 15-21.

Labrie, S. and Moineau, S. (2000). Multiplex PCR for detection and identification of lactococcal bacteriophages. *Appl. Environ. Microbiol.* 66: 987-994.

Labrie, S. and Moineau, S. (2002). Complete genomic sequence of bacteriophage ul36: demonstration of phage heterogeneity within the P335 quasi-species of lactococcal phages. *Virology.* 296: 308-320.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

Lakshmidevi, G., Davidson, B. E. and Hillier, A. J. (1988). Circular permutation of the genome of a temperate bacteriophage from *Streptococcus cremoris* GK5. *Appl. Environ. Microbiol.* 54: 1039-1045.

Lakshmidevi, G., Davidson, B. E. and Hillier, A. J. (1990). Molecular characterization of promoters of the *Lactococcus lactis* subsp. *cremoris* temperate bacteriophage BK5-T and identification of a phage gene implicated in the regulation of promoter activity. *Appl. Environ. Microbiol.* 56: 934-942.

Landoulsi, A., Hughes, P., Kern, R. and Kohiyama, M. (1989). *dam* methylation and the initiation of DNA replication on *oriC* plasmids. *Mol. Gen. Genet.* 216: 217-223.

Lark, K. D. (1972). Evidence for the direct involvement of RNA in the initiation of DNA replication in *Escherichia coli* 15T. *J. Mol. Biol.* 64: 47-60.

Ledeboer, A. M., Bezemer, S., de Haard, J. J. W., Schaffers, I. M., Verrips, C. T., van Vliet, C., Düsterhöft, E.-M., Zoon, P., Moineau, S. and Frenken, L. G. (2002). Preventing phage lysis of *Lactococcus lactis* in cheese production using a neutralizing heavy-chain antibody fragment from llama. *J. Dairy Sci.* 85: 1376-1382.

Lee, D. Y. and Clayton, D. A. (1998). Initiation of mitochondrial DNA replication by transcription and R-loop processing. *J. Biol. Chem.* 273: 30614-30621.

LeGendre, N. and Matsudaira, P. (1988). Direct protein microsequencing from Immobilon-P Transfer Membrane. *BioTechniques* 6: 154-159.

Lemon, K. P. and Grossman, A. D. (1998). Localization of bacterial DNA polymerase: evidence for a factory model of replication. *Science* 282: 1516-1519.

Li, B., Wyman, T. E., Moudgil, T., Marracci, G. H., Ju, C.-F. and Machida, C. A. (1999). Nucleocytoplasmic export of type D Simian retrovirus genomic RNA: identification of important genetic subregions and interacting cellular proteins. *Virology* 264: 37-54.

Li, Z., Reimers, S., Pandit, S. and Deutscher, M. P. (2002). RNA quality control: degradation of defective transfer RNA. *EMBO J.* 21: 1132-1138.

Lillehaug, D., Lindqvist, B. H. and Birkeland, N. K. (1991). Characterization of ϕLC3, a *Lactococcus lactis* subsp. *cremoris* temperate bacteriophage with cohesive single-stranded DNA ends. *Appl. Environ. Microbiol.* 57: 3206-3211.

Lillehaug, D. and Birkeland, N. K. (1993). Characterization of genetic elements required for site-specific integration of the temperate lactococcal bacteriophage phi LC3 and construction of integration-negative phi LC3 mutants. *J. Bacteriol.* 175: 1745-1755.

Lister, J. (1873). A further contribution to the natural history of bacteria and the germ theory of fermentive changes. *Q. Microbiol. Sci.* 13: 380-408.

Liu, L. F. and Wang, J. C. (1987). Supercoiling of the DNA template during transcription. *Proc. Natl. Acad. Sci. USA* 84: 7024-7027.

Lobban, P. E. and Kaiser, A. D. (1973). Enzymatic end-to-end joining of DNA molecules. *J. Mol. Biol.* 78: 453-471.

Loeffler, J. M., Nelson, D. and Fischetti, V. A. (2001). Rapid killing of *Streptococcus pneumoniae* with a bacteriophage cell wall hydrolase. *Science* 294: 2170-2172.

López, C., Navas-Castillo, J., Gowda, S., Moreno, P. and Flores, R. (2000). The 23-kDa protein coded by the 3'-terminal gene of citrus tristeza virus is an RNA-binding protein. *Virology* 269: 462-470.

Lubbers, M. W., Ward, L. J., Beresford, T. P., Jarvis, B. D. and Jarvis, A. W. (1994). Sequencing and analysis of the cos region of the lactococcal bacteriophage c2. *Mol. Gen. Genet.* 245: 160-166.

Lubbers, M. W., Waterfield, N. R., Beresford, T. P., Le Page, R. W. and Jarvis, A. W. (1995). Sequencing and analysis of the prolate-headed lactococcal bacteriophage c2 genome and identification of the structural genes. *Appl. Environ. Microbiol.* 61: 4348-4356.

Lubbers, M. W., Schofield, K., Waterfield, N. R. and Polzin, K. M. (1998). Transcription analysis of the prolate-headed lactococcal bacteriophage c2. *J. Bacteriol.* 180: 4487-4496.

Luria, S. E. and Human, M. L. (1952). A nonhereditary, host-induced variation of bacterial viruses. *J. Bacteriol.* 64: 557-569.

Macrina, F. L., Evans, R. P., Tobian, J. A., Hartley, D. L., Clewell, D. B. and Jones, K. R. (1983). Novel shuttle plasmid vehicles for *Escherichia-Streptococcus* transgeneric cloning. *Gene* 25: 145-150.

Madsen, A. and Josephsen, J. (1998). Cloning and characterization of the lactococcal plasmid-encoded type II restriction/modification system, *Lla*DII. *Appl. Environ. Microbiol.* 64: 2424-2431.

Madsen, P. L., Johansen, A. H., Hammer, K. and Brøndsted, L. (1999). The genetic switch regulating activity of early promoters of the temperate lactococcal bacteriophage TP901-1. *J. Bacteriol.* 181: 7430-7438.

Madsen, S. M., Mills, D., Djordjevic, G., Israelsen, H. and Klaenhammer, T. R. (2001). Analysis of the genetic switch and replication region of a P335-type bacteriophage with an obligate lytic lifestyle on *Lactococcus lactis*. *Appl. Environ. Microbiol.* 67: 1128-1139.

Magni, C., Marini, P. and de Mendoza, D. (1995). Extraction of RNA from gram-positive bacteria. *BioTechniques* 19: 880-884.

Mahanivong, C., Boyce, J. D., Davidson, B. E. and Hillier, A. J. (2001). Sequence analysis and molecular characterization of the *Lactococcus lactis* temperate bacteriophage BK5-T. *Appl. Environ. Microbiol.* 67: 3564-3576.

Maniloff, J. and Ackermann, H. W. (1998). Taxonomy of bacterial viruses: establishment of tailed virus genera and the order Caudovirales [news]. *Arch. Virol.* 143: 2051-2063.

Marians, K. J. (1992). Prokaryotic DNA replication. *Annu. Rev. Biochem.* 61: 673-719.

Masukata, H., Dasgupta, S. and Tomizawa, J. (1987). Transcriptional activation of ColE1 DNA synthesis by displacement of the nontranscribed strand. *Cell* 51: 1123-1130.

Masukata, H. and Tomizawa, J. (1990). A mechanism of formation of a persistent hybrid between elongating RNA and template DNA. *Cell* 62: 331-338.

Mathews, D. H., Sabina, J., Zuker, M. and Turner, D. H. (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J. Mol. Biol.* 288: 911-940.

Matsumoto, J. (1994). Evolutionary role of abortive transcript as a primer for DNA replication. *J. Mol. Evol.* 39: 620-624.

Mattaj, I. W. (1993). RNA recognition: a family matter? *Cell* 73: 837-840.

Matvienko, N. N., Madsen, A. and Josephsen, J. (1999). AbiS, a novel abortive infection mechanism encoded by plasmid pAW601 of *Lactococcus lactis* subsp. *cremoris* W60. NCBI Accession number AJ132009.

Matzura, O. and Wennborg, A. (1996). RNAdraw: an integrated program for RNA secondary structure calculation and analysis under 32-bit Microsoft Windows. *Comp. Appl. Biosci.* 12: 247-249.

McGrath, S., Seegers, J. F., Fitzgerald, G. F. and van Sinderen, D. (1999). Molecular characterization of a phage-encoded resistance system in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 65: 1891-1899.

McGrath, S., Fitzgerald, F. G. and van Sinderen, D. (2001). Improvement and optimization of two engineered phage resistance mechanisms in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 67: 608-616.

McGrath, S., Fitzgerald, G. F. and van Sinderen, D. (2002). Identification and characterization of phage-resistance genes in temperate lactococcal bacteriophages. *Mol. Microbiol.* 43: 509-520.

McLandsborough, L. A., Kolaetis, K. M., Requena, T. and McKay, L. L. (1995). Cloning and characterization of the abortive infection genetic determinant abiD isolated from pBF61 of *Lactococcus lactis* subsp. *lactis* KR5. *Appl. Environ. Microbiol.* 61: 2023-2026.

McLean, M. J., Wolfe, K. H. and Devine, K. M. (1998). Base composition skews, replication orientation, and gene orientation in 12 prokaryote genomes. *J. Mol. Evol.* 47: 691-696.

Melton, D. A., Krieg, P. A., Rebagliati, M. R. R., Maniatis, T., Zinn, K. and Green, M. R. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12: 7035-7056.

Menkens, A. E. and Kreuzer, K. N. (1988). Deletion analysis of bacteriophage T4 tertiary origins. *J. Biol. Chem.* 263: 11358-11365.

Messer, W. and Weigel, C. (1997). DnaA initiator - also a transcription factor. *Mol. Microbiol.* 24: 1-6.

Miao, E. A. and Miller, S. I. (1999). Bacteriophages in the evolution of pathogen-host interactions. *Proc. Natl. Acad. Sci. USA* 96: 8452-8454.

Michel, B. and Ehrlich, S. D. (1986). Illegitimate recombination occurs between the replication origin of the plasmid pC194 and a progressing replication fork. *EMBO J.* 5: 3691-3696.

Miranda, G., Schuppli, D., Barrera, I., Hausherr, C., Sogo, J. M. and Weber, H. (1997). Recognition of bacteriophage Qβ plus strand RNA as a template by Qβ replicase: role of RNA interactions mediated by ribosomal proteins S1 and host factor. *J. Mol. Biol.* 267: 1089-1103.

Moineau, S., Borkaev, M., Holler, B. J., Walker, S. A., Kondo, J. K., Vedamuthu, E. R. and Vandenbergh, P. A. (1996). Isolation and characterization of lactococcal bacteriophages from cultured buttermilk plants in the United States. *J. Dairy Sci.* 79: 2104-2111.

Monteville, M. R., Ardestani, B. and Geller, B. L. (1994). Lactococcal bacteriophages require a host cell wall carbohydrate and a plasma membrane protein for adsorption and ejection of DNA. *Appl. Environ. Microbiol.* 60: 3204-3211.

Mooney, R. A., Artsimovitch, I. and Landick, R. (1998). Information processing by RNA polymerase: recognition of regulatory signals during RNA chain elongation. *J. Bacteriol.* 180: 3265-3275.

Moriya, S., Imai, Y., Hassan, A. K. M. and Ogasawara, N. (1999). Regulation of initiation of *Bacillus subtilis* chromosome replication. *Plasmid* 41: 17-29.

Moscoso, M. and Suárez, J. E. (2000). Characterization of the DNA replication module of bacteriophage A2 and use of its origin of replication as a defense against infection during milk fermentation by *Lactobacillus casei*. *Virology* 273: 101-111.

Mosig, G., Colowick, N., Gruidl, M. E., Chang, A. and Harvey, A. J. (1995). Multiple initiation mechanisms adapt phage T4 DNA replication to physiological changes during T4's development. *FEMS Microbiol. Rev.* 17: 83-98.

Mulugu, S., Potnis, A., Shamsussaman, Taylor, J., Alexander, K. and Bastia, D. (2001). Mechanism of termination of DNA replication of *Escherichia coli* involves helicase-contrahelicase interaction. *Proc. Natl. Acad. Sci. USA* 98: 9569-9574.

Murialdo, H. (1991). Bacteriophage lambda DNA maturation and packaging. *Annu. Rev. Biochem.* 40: 125-153.

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.

Nilsson, D. and Johansen, E. (1994). A conserved sequence in tRNA and rRNA promoters of *Lactococcus lactis*. *Biochim. Biophys. Acta* 1219: 141-144.

O'Connor, L., Coffey, A., Daly, C. and Fitzgerald, G. F. (1996). AbiG, a genotypically novel abortive infection mechanism encoded by plasmid pCI750 of *Lactococcus lactis* subsp. *cremoris* UC653. *Appl. Environ. Microbiol.* 62: 3075-3082.

O'Connor, L., Tangney, M. and Fitzgerald, G. F. (1999). Expression, regulation, and mode of action of the AbiG abortive infection system of *Lactococcus lactis* subsp. *cremoris* UC653. *Appl. Environ. Microbiol.* 65: 330-335.

Ogasawara, N., Fujita, M. Q., Moriya, S., Fukuoka, T., Hirano, M. and Yoskikawa, H. (1990). Comparative anatomy of *oriC* in Eubacteria. *The bacterial chromosome*: 287-295. American Society for microbiology, Washington, D. C.

O'Sullivan, D. J., Hill, C. and Klaenhammer, T. R. (1993). Effect of increasing the copy number of bacteriophage origins of replication, in *trans*, on incoming-phage proliferation. *Appl. Environ. Microbiol.* 59: 2449-2456.

O'Sullivan, D. J. and Klaenhammer, T. R. (1993). High- and low-copy-number *Lactococcus* shuttle cloning vectors with features for clone screening. *Gene* 137: 227-231.

O'Sullivan, D. J., Zagula, K. and Klaenhammer, T. R. (1995). In vivo restriction by *Lla*I is encoded by three genes, arranged in an operon with *llaIM*, on the conjugative *Lactococcus* plasmid pTR2030. *J. Bacteriol.* 177: 134-143.

O'Sullivan, D., Ross, R. P., Twomey, D. P., Fitzgerald, G. F., Hill, C. and Coffey, A. (2001). Naturally occurring lactococcal plasmid pAH90 links bacteriophage resistance and mobility functions to a food-grade selectable marker. *Appl. Environ. Microbiol.* 67: 929-937.

Østergaard, S., Brøndsted, L. and Vogensen, F. K. (2001). Identification of a replication protein and repeats essential for DNA replication of the temperate lactococcal bacteriophage TP901-1. *Appl. Environ. Microbiol.* 67: 774-781.

Parreira, R., Ehrlich, S. D. and Chopin, M. C. (1996). Dramatic decay of phage transcripts in lactococcal cells carrying the abortive infection determinant AbiB. *Mol. Microbiol.* 19: 221-230.

Pedersen, M., Østergaard, S., Bresciani, J. and Vogensen, F. K. (2000). Mutational analysis of two structural genes of the temperate lactococcal bacteriophage TP901-1 involved in tail length determination and baseplate assembly. *Virology* 276: 315-328.

Peeters, B. P. H., de Boer, J. H., Bron, S. and Venema, G. (1988). Structural plasmid instability in *Bacillus subtilis*: effect of direct and inverted repeats. *Mol. Gen. Genet.* 212: 450-458.

Perez-Casal, J., Caparon, M. G. and Scott, J. R. (1991). Mry, a *trans*-acting positive regulator of the M protein gene of *Streptococcus pyogenes* with similarity to the receptor proteins of two-component regulatory systems. *J. Bacteriol.* 173: 2617-2624.

Perrin, R., Billard, P. and Branlant, C. (1997). Comparative analysis of the genomic DNA terminal regions of the lactococcal bacteriophages from species c2. *Res. Microbiol.* 148: 573-583.

Petersen, A., Josephsen, J. and Johnsen, M. G. (1999). TPW22, a lactococcal temperate phage with a site-specific integrase closely related to *Streptococcus thermophilus* phage integrases. *J. Bacteriol.* 181: 7034-7042.

Pillidge, C. J. and Jarvis, A. W. (1988). DNA restriction maps and classification of the lactococcal bacteriophages c2 and sk1. *N. Z. J. Dairy Sci. Technol.* 23: 411-416.

Piñol-Roma, S., Adam, S. A., Choi, Y. D. and Dryfuss, G. (1989). Ultraviolet-induced cross-linking of RNA to proteins *in vivo*. *Methods Enzymol.* 180: 410-418.

Platteeuw, C. and de Vos, W. M. (1992). Location, characterization and expression of lytic enzyme-encoding gene, *lytA*, of *Lactococcus lactis* bacteriophage phi US3. *Gene* 118: 115-120.

Polzin, K. M., Collins, L. J. and Lubbers, M. W. (1999). Protecting dairy starters from bacteriophage infection: development of novel resistance mechanisms against lactococcal prolate bacteriophage c2. *Recent Res. Devel. Microbiol.* 3: 125-133.

Postow, L., Crisona, N. J., Peter, B. J., Hardy, C. D. and Cozzarelli, N. R. (2001). Topological challenges to DNA replication: conformations at the fork. *Proc. Natl. Acad. Sci.* 98: 8219-8226.

Powell, I. B. and Davidson, B. E. (1986). Resistance to *in vitro* restriction of DNA from lactic streptococcal bacteriophage c6A. *Appl. Environ. Microbiol.* 51: 1358-1360.

Powell, I. B., Arnold, P. M., Hillier, A. J. and Davidson, B. E. (1989). Molecular comparison of prolate- and isometric-headed bacteriophages of lactococci. *Can. J. Microbiol.* 35: 860-866.

Powell, I. B, Tulloch, D. L., Hillier, A. J. and Davidson, B. E. (1992). Phage DNA synthesis and host DNA degradation in the life cycle of *Lactococcus lactis* bacteriophage c6A. *J. Gen. Microbiol.* 138: 945-950.

Prentki, P. and Krisch, H. M. (1984). *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* 29: 303-313.

Prévots, F., Mata, M. and Ritzenthaler, P. (1990). Taxonomic differentiation of 101 lactococcal bacteriophages and characterization of bacteriophages with unusually large genomes. *Appl. Environ. Microbiol.* 56: 2180-2185.

Prévots, F., Daloyau, M., Bonin, O., Dumont, X. and Tolou, S. (1996). Cloning and sequencing of the novel abortive infection gene *abiH* of *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* S94. *FEMS Microbiol. Lett.* 142: 295-299.

Prévots, F. and Ritzenthaler, P. (1998). Complete sequence of the new lactococcal abortive phage resistance gene *abiO*. *J. Dairy Sci.* 81: 1483-1485.

Prévots, F., Tolou, S., Delpech, B., Kaghad, M. and Daloyau, M. (1998). Nucleotide sequence and analysis of the new chromosomal abortive infection gene *abiN* of *Lactococcus lactis* subsp. *cremoris* S114. *FEMS Microbiol. Lett.* 159: 331-336.

Ptashne, M. (1967). Specific binding of the λ phage repressor to λ DNA. *Nature* 214: 232-234.

Qin, M.-H., Madiraju, M. V. V. S. and Rajagopalan, M. (1999). Characterization of the functional replication origin of *Mycobacterium tuberculosis*. *Gene* 233: 121-130.

Rakonjac, J., Ward, L. J. H., Schiemann, A. H., Gardner, P. P., Lubbers, M. W. and O'Toole, P. W. (2003). Sequence diversity and functional conservation of the origin of replication in lactococcal prolate phages. *Appl. Environ. Microbiol.* 69: 5104-5114.

Régnier, P. and Arraiano, C. M. (2000). Degradation of mRNA in bacteria: emergence of ubiquitous features. *BioEssays* 22: 235-244.

Relano, P., Mata, M., Bonneau, M. and Ritzenthaler, P. (1987). Molecular characterization and comparison of 38 virulent and temperate bacteriophages of *Streptococcus lactis*. *J. Gen. Microbiol.* 133: 3053-3063.

Ringquist, S., Jones, T., Snyder, E. E., Gibson, T., Boni, I. and Gold, L. (1995). High-affinity RNA ligands to *Escherichia coli* ribosomes and ribosomal protein S1: comparison of natural and unnatural binding sites. *Biochemistry* 34: 3640-3648.

Rivas, E. and Eddy, S. R. (1999). A dynamic programming algorithm for RNA structure prediction including pseudoknots. *J. Mol. Biol.* 285: 2053-2068.

Roberts, J. W. (1969). Termination factor for RNA synthesis. *Nature* 224: 1168-1174.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual. 2nd Edition. *Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.*

Sanger, F., Donelson, J. E., Coulson, A. R., Kossel, H. and Fischer, D. (1974). Determination of a nucleotide sequence in bacteriophage f1 DNA by primed synthesis with DNA polymerase. *J. Mol. Biol.* 90: 315-333.

Sasse-Dwight, S. and Gralla, J. D. (1989). $KMnO_4$ as a probe for *lac* promoter DNA melting and mechanism *in vivo*. *J. Biol. Chem.* 264: 8074-8081.

Saxelin, M.-L., E.-L., N.-K., Merilainen, V. T. and Forsen, R. I. (1986). Ultrastructure and host specificity of bacteriophages of *Streptococcus cremoris*, *Streptococcus lactis* subsp. *diacetylactis*, and *Leuconostoc cremoris* from finnish fermented milk "viili". *Appl. Environ. Microbiol.* 52: 771-777.

Schleifer, K. H. (1987). Recent changes in the taxonomy of lactic acid bacteria. *Fed. Eur. Microbiol. Soc. Microbiol. Rev.* 46: 201.

Schnier, J. and Isono, K. (1982). The DNA sequence of the gene *rpsA* of *Escherichia coli* coding for ribosomal protein S1. *Nucleic Acids Res.* 10: 1857-1865.

Schouler, C., Bouet, C., Ritzenthaler, P., Drouet, X. and Mata, M. (1992). Characterization of *Lactococcus lactis* phage antigens. *Appl. Environ. Microbiol.* 58: 2479-2484.

Schouler, C., Ehrlich, S. D. and Chopin, M. C. (1994). Sequence and organization of the lactococcal prolate-headed bIL67 phage genome. *Microbiology* 140: 3061-3069.

Schouler, C., Gautier, M., Ehrlich, S. D. and Chopin, M. C. (1998a). Combinational variation of restriction modification specificities in *Lactococcus lactis. Mol. Microbiol.* 28: 169-178.

Schouler, C., Clier, F., Lerayer, A. L., Ehrlich, S. D. and Chopin, M. C. (1998b). A type IC restriction-modification system in *Lactococcus lactis. J. Bacteriol.* 180: 407-411.

Sengupta, J., Agrawal, R. K. and Frank, J. (2001). Visualization of protein S1 within the 30S ribosomal subunit and its interaction with messenger RNA. *Proc. Natl. Acad. Sci. USA* 98: 11991-11996.

Shearman, C., Underwood, H., Jury, K. and Gasson, M. (1989). Cloning and DNA sequence analysis of a *Lactococcus* bacteriophage lysin gene. *Mol. Gen. Genet.* 218: 214-221.

Siezen, R. J. (1999). Multi-domain, cell-envelope proteinases of lactic acid bacteria. *Antonie van Leeuwenhoek* 76: 139-155.

Sijtsma, L., Sterkenburg, A. and Wouters, J. T. M. (1988). Properties of the cell walls of *Lactococcus lactis* subsp. *cremoris* SK110 and SK112 and their relation to bacteriophage resistance. *Appl. Environ. Microbiol.* 54: 2808-2811.

Sijtsma, L., Jansen, N., Hazeleger, W. C., Wouters, J. T. M. and Hellingwerf, K. J. (1990). Cell surface characteristics of bacteriophage-resistant *Lactococcus lactis* subsp. *cremoris* SK110 and its bacteriophage-sensitive variant SK112. *Appl. Environ. Microbiol.* 56: 3230-3233.

Smith, G. P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 228: 1315-1317.

Sørensen, M. A., Fricke, J. and Pedersen, S. (1998). Ribosomal protein S1 is required for translation of most, if not all, natural mRNAs in *Escherichia coli in vivo. J. Mol. Biol.* 280: 561-569.

Spagnolo, J. F. and Hogue, B. G. (2000). Host protein interactions with the 3' end of bovine Coronavirus RNA and the requirement of the poly(A) tail for Coronavirus defective genome replication. *J. Virol.* 74: 5053-5065.

Speck, C. and Messer, W. (2001). Mechanism of origin unwinding: sequential binding of DnaA to double- and single-stranded DNA. *EMBO J.* 20: 1469-1476.

Studier, F. W. (1973). Genetic analysis of essential bacteriophage T7 genes. *J. Mol. Biol.* 79: 227-236.

Sturino, J. M. and Klaenhammer, T. R. (2002). Expression of antisense RNA targeted against *Streptococcus thermophilus* bacteriophages. *Appl. Environ. Microbiol.* 68: 588-596.

Su, P., Harvey, M., Im, H. J. and Dunn, N. W. (1997). Isolation, cloning and characterisation of the *abiI* gene from *Lactococcus lactis* subsp. *lactis* M138 encoding abortive phage infection. *J. Biotech.* 54: 95-104.

Subramanian, A.-R. (1983). Structure and functions of ribosomal protein S1. *Prog. Nucleic Acids Res. Mol. Biol.* 28: 101-142.

Sugimoto, K., Kohara, Y. and Okazaki, T. (1987). Relative roles of T7 RNA polymerase and gene *4* primase for the initiation of T7 phage DNA replication *in vivo*. *Proc. Natl. Acad. Sci. USA* 84: 3977-3981.

Sutton, M. D. and Walker, G. C. (2001). Managing DNA polymerases: coordinating DNA replication, DNA repair, and DNA recombination. *Proc. Natl. Acad. Sci. USA* 98: 8342-8349.

Symmons, M. F., Williams, M. G., Luisi, B. F., Jones, G. H. and Carpousis, A. J. (2002). Running rings around RNA: A superfamily of phosphate-dependent RNases. *Trends Biochem. Sci.* 27:11-18.

Szalewska-Palasz, A., Wegrzyn, A., Blaszczak, A., Taylor, K. and Wegrzyn, G. (1998). DnaA-stimulated transcriptional activation of *ori* λ: *Escherichia coli* RNA polymerase β subunit as a transcriptional activator contact site. *Proc. Natl. Acad. Sci USA* 95: 4241-4246.

Taylor, K. and Wegrzyn, G. (1995). Replication of coliphage lambda DNA. *FEMS Microbiol. Rev.* 17: 109-119.

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

Terzaghi, B. E. (1976). Morphologies and host sensitivities of lactic streptococcal phages from cheese factories. *N. Z. J. Dairy Sci. Technol.* 11: 155-163.

Teuber, M. and Lembke, J. (1983). The bacteriophages of lactic acid bacteria with emphasis on genetic aspects of group N lactic streptococci. *Antonie van Leeuwenhoek* 49: 283-295.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673-4680.

Twomey, D. P., de Urraza, P. J., McKay, L. L. and O'Sullivan, D. J. (2000). Characterization of AbiR, a novel multicomponent abortive infection mechanism encoded by plasmid pKR223 of *Lactococcus lactis* subsp. *lactis* KR2. *Appl. Environ. Microbiol.* 66: 2647-2651.

Valyasevi, R., Sandine, W. E. and Geller, B. L. (1990). The bacteriophage kh receptor of *Lactococcus lactis* subsp. *cremoris* KH is the rhamnose of the extracellular wall polysaccharide. *Appl. Environ. Microbiol.* 56: 1882-1889.

Valyasevi, R., Sandine, W. E. and Geller, B. L. (1991). A membrane protein is required for bacteriophage c2 infection of *Lactococcus lactis* subsp. *lactis* C2. *J. Bacteriol.* 173: 6095-6100.

van de Guchte, M., Daly, C., Fitzgerald, G. F. and Arendt, E. K. (1994). Identification of the putative repressor-encoding gene *cI* of the temperate lactococcal bacteriophage Tuc2009. *Gene* 144: 93-95.

van Sinderen, D., Karsens, H., Kok, J., Terpstra, P., Ruiters, M. H. J., Venema, G. and Nauta, A. (1996). Sequence analysis and molecular characterization of the temperate lactococcal bacteriophage r1t. *Mol. Microbiol.* 19: 1343-1355.

Volkin, E. and Astrachan, L. (1956). Phosphorus incorporation in *Escherichia coli* ribonucleic acid after infection with bacteriophage T2. *Virology* 2: 149-161.

Waldor, M. K. and Mekalanos, J. J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* 272: 1910-1914.

Walker, S. A. and Klaenhammer, T. R. (1998). Molecular characterization of a phage-inducible middle promoter and its transcriptional activator from the lactococcal bacteriophage ϕ31. *J. Bacteriol.* 180: 921-931.

Walker, S. A., and Klaenhammer, T. R. (2000). An explosive antisense RNA strategy for inhibition of a lactococcal bacteriophage. *Appl. Environ. Microbiol.* 66: 310-319.

Walter, A. E., Turner, D. H., Kim, J., Lyttle, M. H., Müller, P., Mathews, D. H. and Zuker, M. (1994). Coaxial stacking of helixes enhances binding of oligoribonucleotides and improves predictions of RNA folding. *Proc. Natl. Acad. Sci. USA* 91: 9218-9222.

Wang, I.-N., Smith, D. L. and Young, R. (2000). Holins: the protein clocks of bacteriophage infections. *Annu. Rev. Microbiol.* 54: 799-825.

Ward, L. J., Beresford, T. P., Lubbers, M. W., Jarvis, B. D. and Jarvis, A. W. (1993). Sequence analysis of the lysin gene region of the prolate lactococcal bacteriophage c2. *Can. J. Microbiol.* 39: 767-774.

Waterfield, N. R., Lubbers, M. W., Polzin, K. M., Le Page, R. W. and Jarvis, A. W. (1996). An origin of DNA replication from *Lactococcus lactis* bacteriophage c2. *Appl. Environ. Microbiol.* 62: 1452-1453.

Weigel, C., Messer, W., Preiss, S., Welzeck, M., Boye, M. and Boye, E. (2001). The sequence requirements for a functional *Escherichia coli* replication origin are different for the chromosome and a minichromosome. *Mol. Microbiol.* 40: 498-507.

Weisblum, B. (1995). Erythromycin resistance by ribosome modification. *Antimicrob. Agents Chemother.* 39: 577-585.

Whitehead, H. R. and Cox, G. A. (1935). The occurrence of bacteriophage in cultures of lactic streptococci. *N. Z. J. Dairy Sci. Technol.* 16: 319-320.

Whitman, W. B., Coleman, D. C. and Wiebe, W. J. (1998). Prokaryotes: the unseen majority. *Proc. Natl. Acad. Sci. USA.* 95: 6578-6583.

Xia, T., SantaLucia, J., Jr., Burkard, M. E., Kierzek, R., Schroeder, S. J., Jiao, X., Cox, C. and Turner, D. H. (1998). Thermodynamic parameters for an expanded nearest-neighbor model for formation of RNA duplexes with Watson-Crick base pairs. *Biochemistry* 37: 14719-14735.

Xu, F. F., Pearce, L. E. and Yu, P. L. (1990). Molecular cloning and expression of a proteinase gene from *Lactococcus lactis* subsp. *cremoris* H2 and construction of a new lactococcal vector pFX1. *Arch. Microbiol.* 154: 99-104.

Xu, F. F., Pearce, L. E. and Yu, P. L. (1991a). Construction of a family of lactococcal vectors for gene cloning and translational fusion. *FEMS Microbiol. Lett.* 61: 55-59

Xu, F. F., Pearce, L. E. and Yu, P. L. (1991b). Genetic analysis of a lactococcal plasmid replicon. *Mol. Gen. Genet.* 227: 33-39.

Xu, F., Lin-Chao, S. and Cohen, S. N. (1993). The *Escherichia coli pcnB* gene promotes adenylylation of antisense RNAI of ColE1-type plasmids *in vivo* and degradation of RNAI decay intermediates. *Proc. Natl. Acad. Sci. USA.* 90: 6756-6760.

Yanish-Perron, C., Vieira, J. and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33: 103-119.

Yates, J. L., Camiolo, S. M. and Bashaw, J. M. (2000). The minimal replicator of Epstein-Barr virus *oriP*. *J. Virol.* 74: 4512-4522.

Young, R., Wang, I.-N. and Roof, W. (2000). Phages will out: strategies of host cell lysis. *Trends Microbiol.* 8: 120-128.

Zúñiga, M., Franke-Fayard, B., Venema, G., Kok, J. and Nauta, A. (2002). Characterization of the putative replisome organizer of the lactococcal bacteriophage r1t. *J. Virol.* 76: 10234-10244.