Analysis of Three Phage Resistance Mechanisms and a *recA* Homologue Encoded by the Lactococcal Plasmid pNP40

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For my parents,
and for Vinny

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _Patricia Gawey_   Date:_14/2/95_

Candidate

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Abstract

Phage represent a major problem in dairy fermentations Investigation of the phage resistance mechanisms employed by naturally insensitive lactococcal strains should aid in the development of rational strategies to help combat the problem

The lactococcal plasmid pNP40 from *Lactococcus lactis* ssp *lactis* biovar *diacetylactis* confers complete resistance to the prolate-headed øc2 and the small isometric-headed ø712 in *L lactis* ssp *lactis* MG1614, and has been used successfully to construct commercially valuable starter strains In this study, the determinants for two independent abortive infection phage resistance systems (AbiE and AbiF) were cloned AbiF was shown to act at the level of phage DNA replication while AbiE operates post-replication, possibly at the level of transcription/translation or phage packaging/release pNP40 was also found to mediate resistance to øc2 by a novel early-acting phage resistance mechanism which we propose prevents phage DNA penetration into the host

Sequence analysis of the determinants for AbiE and AbiF demonstrated that two overlapping ORF's, of 861 bp and 894 bp respectively, are required for expression of AbiE while a single 1026 bp ORF encodes AbiF Two ORF's are located between the *abiE* and *abiF* determinants, one of which codes for a RecA homologue This represents, to our knowledge, the first *recA* gene located on a plasmid Using a RecA deficient lactococcal strain, it was established that while the chromosomally-encoded RecA is required for full phenotypic expression of AbiF, the pNP40-encoded RecA has no discernible role in phage resistance Thus, pNP40-directed phage insensitivity is mediated by three independent phage resistance systems AbiE, AbiF and a novel phage DNA penetration blocking mechanism

CHAPTER I

BACTERIOPHAGE RESISTANCE IN *LACTOCOCCUS*

1.1 INTRODUCTION

Bacteriophage (phage) interference in dairy fermentations has been recognised since the mid 1930's (Whitehead and Cox, 1935) Slow acid production as a consequence of bacteriophage infection can result in an inferior grade product or even complete starter failure The consequent cost to the dairy industry in terms of time, effort and money has provided a powerful stimulus for research into many aspects of phage-host interactions The areas which have received most attention include the classification of phages, phage-relatedness of starter cultures, the lytic and lysogenic cycles of phage, molecular characterisation of phage genomes and the analysis of host-encoded defence against phage proliferation

Precautions commonly taken to minimise phage-related disruptions in dairy plants include closed vat systems, the development of concentrated starter cultures for direct inoculation and the practice of propagating the bulk culture in phage inhibitory media (for reviews, see Klaenhammer, 1987 and Cogan *et al*, 1991) Starter culture rotation (Keogh, 1972) and the use of multiple strain-starters of phage-unrelated strains (Thunell *et al*, 1981, Daniell and Sandine, 1981) are additional strategies which are widespread in the dairy industry

Recognition of the potential of bacteriophage insensitive mutants (BIM's) followed almost immediately upon the identification of phage as a source of problems in dairy fermentations Strains which succumbed to phage attack were substituted in subsequent fermentations by insensitive derivatives isolated following repeated subculture in the presence of infective phage (Limsowtin and Terzaghi, 1976, Jarvis, 1981, Thunell *et al*, 1984, Marshall and Berridge, 1976) However, these mutants were frequently found to revert to phage sensitivity or to succumb to attack by alternative phage Moreover, difficulties were encountered in the isolation of phage resistant mutants which retained the ability to ferment lactose and utilise casein (Hull,

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1983), and this led to the development of media such as fast-slow differential agar and milk citrate agar to identify isolates displaying the desired metabolic traits BIM's have been used successfully on a long-term basis in a limited number of instances (Jarvis, 1981, Thunell *et al*, 1981), however, they are generally regarded as a short-term solution (Klaenhammer, 1987)

The natural phage insensitivity of a number of starter cultures has been observed for some time The development of sophisticated genetic and molecular technologies for lactococci has established that these bacteria can harbour a battery of resistance mechanisms which are usually, but not always, plasmid-encoded The plasmid location of these systems had initially been suspected because of the instability of resistance phenotypes (Limsowtin *et al*, 1978) although conclusive evidence was not obtained until 1981 when Sanders and Klaenhammer correlated the loss of phage resistance by a derivative of *Lactococcus lactis* ssp *lactis* KH with the disappearance of the plasmid pME100 Since this initial observation, numerous lactococcal phage resistance plasmids have been identified, several of which were found to be conjugative (Klaenhammer and Sanozsky, 1985, McKay and Baldwin, 1984, Baumgartner *et al*, 1986, Coffey *et al*, 1989) This permitted their introduction *via* conjugal strategies into starter strains, increasing the range of phage resistant cultures available for commercial applications (Sanders *et al*, 1986, Jarvis, 1988, Harrington and Hill, 1991, Kelly *et al*, 1990) Extensive studies on the mechanisms of phage resistance have enabled their classification into three principal categories adsorption inhibition, restriction/modification (R/M) and abortive infection (Abi) This chapter reviews recent molecular and mechanistic investigations into the nature of these phage resistance systems, and also details exciting new developments regarding phage evolution as a result of the introduction of conjugative phage resistance plasmids into commercial strains

1.2 THE BACTERIOPHAGE LYTIC CYCLE

Any meaningful review of phage resistance requires a thorough appreciation of the sequence of events which occurs during phage infection This includes the requirements for phage attachment and the nature of bacteriophage receptors of lactococci In addition, the subsequent intracellular development of progeny phage particles facilitates identification of the mechanisms by which phage proliferation may be inhibited

1.2.1 Early phage infection

The most complete study of early phage infection in lactococci has been conducted by Geller and co-workers (Valyasevi *et al* , 1991, Valyasevi *et al* , 1994, Geller *et al* , 1993 and Monteville *et al* , 1994) on the adsorption to and phage DNA injection into *L lactis* ssp *lactis* C2 by phage øc2 The initial interaction involves attachment of the phage to carbohydrate moieties in the cell wall The initial adsorption stage was found to be reversible following the addition of L-rhamnose suggesting a rhamnosyl compound as the phage receptor but this binding became irreversible over time as infection continued The phage then appeared to interact with a specific membrane-located protein, PIP (phage infection protein), as membranes purified from strain C2 derivatives deficient in PIP failed to adsorb phage (Valyasevi *et al* , 1991) This adsorption to the membrane was shown to be independent of temperature, however, an injection study in which phages with tritium-labelled DNA were adsorbed to purified membranes demonstrated that the subsequent DNA ejection step was temperature dependent At 4°C or 30°C, tritium-labelled DNA became associated with purified membranes but subsequent disassociation, presumably as a consequence of translocation of the phage DNA across the membrane, was only observed at 30°C

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(Monteville *et al.*, 1994). A 2.7 kb chromosomal gene from *L. lactis* ssp. *lactis* C2 was found to complement the PIP deficient derivative restoring phage sensitivity (Geller *et al.*, 1993). In addition, a 32 kDa protein which possessed the ability to inactivate phage was purified from the membrane fraction of *L. lactis* ssp. *lactis* C2 cells (Valyasevi *et al.*, 1991). The involvement of PIP or the 32 kDa protein in translocation of the phage DNA across the membrane remains to be established. In addition, six other lactococcal phages were shown to share the same receptor sites as phage c2 both on the cell wall and the cell membrane (Monteville *et al.*, 1994).

Although carbohydrate cell wall components, in particular L-rhamnose, have been implicated as receptors for many other phage (Schafer *et al.*, 1991; Valyasevi *et al.*, 1990; Keogh and Pettinghill, 1983), the involvement of the cell membrane has only been demonstrated in the case of øml3 (Oram, 1971) and possibly also øeb7 (Keogh and Pettinghill, 1983). In fact, Valyasevi *et al.* (1991) have shown that øsk1 is not inactivated by cell membranes isolated from *L. lactis* ssp. *lactis* C2, indicating that different lactococcal phages have alternative routes for adsorption and DNA injection into the host. This was also demonstrated by the pattern of adsorption of lactococcal phages to their hosts; Budde-Niekiel and Teuber (1987) and Schafer *et al.* (1991) both observed that adsorption could occur either to specific locations or uniformly over the entire cell surface.

In *Lactobacillus casei*, L-rhamnose and D-glucose were also identified as inhibitors of phage adsorption, e.g. øPL-1 (Watanabe and Takesue, 1975) and analysis of a selection of L-rhamnosyl derivatives examined for their ability to competitively inhibit phage adsorption suggested methyl-O-α-L-rhamnopyranosyl-α-L-rhamnoside as the most closely related of the compounds tested to the chemical structure of the receptor site for PL-1 phage on the cell wall of *Lb. casei* (Watanabe *et al.*, 1992). While the role of the cell membrane in DNA injection was not investigated, this process was not only shown to be calcium- (Watanabe and Takesue, 1972) and temperature-dependent (Watanabe and Takesue, 1975) but to be inhibited by protein synthesis

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inhibitors such as chloramphenicol and erythromycin (Watanabe *et al*, 1991) It was proposed that proteins synthesized during the early stages of infection and prior to complete DNA ejection itself were required for transfer of DNA from øPL-1 to cells of *Lb casei*

1.2 2 Intracellular phage development

Several studies have investigated the intracellular development of phage during the lytic cycle If no restriction endonucleases are encountered, or if they are eluded through the modification of the infecting phage DNA, shut-down of the host metabolism is mediated by early phage genes and phage replication is initiated Powell *et al* (1992) demonstrated that infection of *L lactis* ssp *lactis* C6 by øc6A resulted in inhibition of culture growth within ten minutes, reflecting cell killing by the phage Assays using ^{3}H-labelled phage DNA showed that degradation of host DNA began within six minutes of infection and that the breakdown products were incorporated into progeny øc6A DNA Quantitative DNA hybridizations indicated that synthesis of phage DNA began within six minutes of infection and continued at an approximately constant rate throughout the latent period ø31 was shown by Hill *et al* (1991a) to replicate by a concatemeric intermediate and evidence was supplied to suggest a similar mechanism for øc6A (Powell *et al*, 1992)

Transcription studies conducted with øTuc2009, a temperate phage from *L lactis* ssp *cremoris* UC509, have permitted construction of a transcription map outlining the sequential expression of the phage genome Casey (1991) described the orderly transcription of the øTuc2009 genome beginning with early gene expression and progressing to the distal portion of the genome where the late genes appeared to be clustered

A study by Beresford *et al* (1993) made similar observations for the lactococcal phages øc2 and øsk1 Hybridisation analysis using cDNA, synthesised from mRNA

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isolated at increasing time intervals from a øc2-infected host and probed against digested øc2 DNA showed that transcription of øc2 was temporally regulated Early phage genes composed about one third of the phage genome with the remainder encoding late phage genes, including the phage structural genes and the lysin gene The authors also observed a variation in the level of transcription of early and late phage genes Early phage genes were apparently transcribed at a higher rate and it was suggested that this was due to the necessity for early transcripts to compete with host transcripts No decrease was observed in the number of early øc2 transcripts as the lytic cycle progressed

Transcription of øsk1, however, followed three stages early, middle and late (Beresford *et al*, 1993) with early transcripts being replaced progressively by middle and late transcripts Subsequently, Chandry *et al* (1994) provided a more detailed transcription map for øsk1 Total RNA was isolated from lactococcal cells harvested at various time intervals following infection with øsk1 and separated by electrophoresis Northern blots of these gels probed with specific øsk1 DNA fragments confirmed that transcription occured in three stages early (2-5 minutes following infection), middle (7-10 minutes following infection), and late (at least 15 minutes following infection) Seven partially overlapping early transcripts were detected which were transcribed from a 10 kb region of the phage genome Nine overlapping middle transcripts covering 2 kb of the phage genome were divergently transcribed relative to the early genes and four transcripts were detected from a 16 kb region corresponding to the late transcribed genes Early and late transcripts terminated at a common location on the phage genome The authors proposed that the overlapping nature of the transcripts detected suggested that post-transcriptional processing of the RNA occurred to facilitate differential gene expression from a limited number of promoters

More recently, sequencing of the entire genome of the lactococcal prolate-headed phage bIL67 (Schouler *et al*, 1994) and other lactococcal phages, e g øTuc2009 (van de Guchte *et al*, 1993) and øR1-t (Nauta *et al*, 1993), has provided

valuable information on the organisation of phage genomes Thirty seven ORF's were identified on the genome of øbIL67 which were organised into two clusters One cluster comprised 17 ORF's, one of which showed features in common with a DNA polymerase and another whose deduced amino acid sequence demonstrated significant homology to a recombinase Late genes such as the lysin gene, a minor tail protein, a putative holin and a terminase subunit were identified in the second gene cluster The two gene clusters were divergently transcribed and terminated at the phage *cos* site Interestingly, two sub-clusters comprising ORFs 4, 5 and 6 and ORFs 12 and 13 were flanked by repeated sequences of 25 bp and 31 bp, respectively The authors suggested that these features may be involved in Campbell-type recombination and exchange of phage modules This information may explain the highly recombinogenic properties exhibited by some phages Sequence analysis of the temperate lactococcal phages øR1-t (Nauta *et al* , 1993) and øTuc2009 (van de Guchte *et al* , 1993) have demonstrated the widespread presence of translationally coupled genes confirming the compact nature of phage genomes In addition to identifying particular structural proteins (Arendt *et al* , 1994), van de Guchte *et al* (1994) detected a protein which demonstrated homology to a *Staphlyococcus aureus* phage øL54a integrase

Electron microscopic studies by Watanabe *et al* (1990) provided visual evidence of phage assembly and host lysis during infection of *Lb casei* ATCC 27092 by øPL-1 Sixty minutes following infection, phage head particles began to appear at random in the nuclear region At 90 minutes these particles appeared to cluster, some more dense than others reflecting the presence of phage DNA in the phage heads At the end of the latent period, the cells were ruptured at several points in the cell wall, presumably by a phage-encoded lysin, releasing progeny phage Mature phage particles were observed clustered together within the disintegrating host and dispersed outside

Latent periods have been found to vary considerably between 9 and 139 minutes for different lactococcal phages and burst sizes of up to 250 particles per cell have been recorded, however, these figures varied depending on the temperature of

infection and in some instances, on the propagating host (Keogh, 1973, Sing and Klaenhammer, 1990, Klaenhammer and Fitzgerald, 1994)

1 3   ADSORPTION INHIBITION MECHANISMS

Adsorption inhibition may occur in either of two ways the phage receptor may be absent, altered or masked by substances produced by the resistant cell Masking of the receptor can in some instances be reversed by removal of the masking material, whereas phage adsorption ability cannot be restored to a strain where a mutation is responsible for the resistance phenotype

1.3.1 Bacteriophage insensitive mutants

As soon as phage were identified in dairy fermentations, the value of isolating insensitive mutants was recognised Considerable research efforts were focussed on the isolation of resistant mutants which retained the ability to produce acid at normal rates (Limsowtin and Terzaghi, 1976, Thunell et al, 1984) The majority of mutants did not adsorb phage although the specific molecular events resulting in the resistant phenotype were generally not characterised Resistance was believed to have arisen from single mutations in the genes responsible for the phage receptors, as the rate of accumulation of phage resistant variants was similar to that expected for point or frame shift mutations (King et al, 1983)

Harrington and Hill (1992), however, have linked a plasmid cointegration event with the appearance of a bacteriophage insensitive mutant of L lactis ssp lactis biovar diacetylactis DPC220 Two plasmids present in the parent strain were replaced in the mutant, DPC721, by a novel plasmid pAH90 which was shown to be a discreet cointegrate of the original plasmids The presence of pAH90 in L lactis ssp lactis

MG1614 inhibits adsorption of its homologous phage øc2 The authors proposed that the adsorption inhibition phenotype was activated by the cointegration event, as neither pAH33 nor pAH82 (the original plasmids) conferred this phenotype Systems such as this may represent an undetected pool of genetic material which remains dormant until new phage are encountered Genetic analysis of this novel system will enhance our understanding of the control exercised by the cell in phage management

Coventry *et al* (1984) also observed altered plasmid profiles for some of their adsorption deficient mutants, however, no attempt was made to correlate the putative rearrangements with altered phage sensitivity Neither was it established whether the phage resistant derivatives, which differed with respect to plasmid content, were contaminating lactic acid bacteria isolated during the selection process or true bacteriophage insensitive mutants

1.3.2 Plasmid-encoded adsorption inhibition

Plasmids encoding adsorption inhibition have been isolated from several phage insensitive strains (Table 1 1) Sanders and Klaenhammer (1983) were the first to identify an adsorption blocking plasmid when several phage sensitive variants of *L lactis* ssp *lactis* ME2 were discovered to be lacking a 30 MDa plasmid designated pME0030 Absence of this plasmid enabled four phage to adsorb more efficiently to this host

The most extensively studied plasmid-encoded adsorption inhibition mechanism is that determined by pSK112, a 34 MDa plasmid from *L lactis* ssp *lactis* SK110 (de Vos *et al* , 1984) Native SK110 failed to adsorb phage whereas derivatives cured of pSK112 adsorbed phage normally Alkali treatment of pSK112-containing hosts also permitted the phage adsorption process to proceed normally Sijtsma *et al* (1988) proposed that phage resistance was mediated through shielding of the phage receptor by a galactose-containing layer since material recovered following the alkali treatment was

Table 1.1

Lactococcal plasmids which encode phage resistance mechanisms which interfere with phage adsorption

Plasmid	Size(kb)	Source strain		Original Reference
pME0030	46	LL	ME2	Sanders and Klaenhammer (1983)
pSK112	52	LC	SK110	de Vos *et al* (1984)
pCI528	46	LC	UC503	Costello (1988)
pKC50	80	LL	57150	Tortorello *et al* (1990)
pAH90	90	LD	DPC220	Harrington and Hill (1992)
P2520L	37 5	LL	P25	Akcelik and Tunail (1992)
p1149-3	12 8	LC	11/49	Geis *et al* (1987)

LL -*L lactis* ssp *lactis*

LC -*L lactis* ssp *cremoris*

LD -*L lactis* ssp *lactis* biovar *diacetylactis*

identified as a galactose-containing lipoteichoic acid or a part thereof (Sijtsma et al ,

1990a) Lipoteichoic acid extracted from the resistant host was also found to contain a

higher lipid lipoteichoic acid ratio than the sensitive derivative, and this resulted in a

more hydrophobic cell surface (Sijtsma et al , 1990b)

pCI528, an adsorption blocking plasmid identified by Costello (1988) mediates

resistance by a mechanism similar to that of pSK112 Originally detected in L lactis

ssp cremoris UC503, this 46 kb plasmid altered the cell surface hydrophobicity of its

host Electron microscopy studies demonstrated the presence of an irregularly

distributed substance surrounding the cell surface (Lucey et al , 1992) As was the case

with pSK112, dilute alkali washing of resistant cells restored full sensitivity to phage

Gas liquid chromotography (GLC) analysis revealed elevated levels of galactose and

rhamnose in cell wall samples of resistant strains The authors concluded that phage

adsorption was inhibited by the production of a hydrophilic polymer containing both

rhamnose and galactose which shielded receptor sites

Two other plasmids providing phage resistance by adsorption inhibition have

been identified Tortorello et al (1990) attributed adsorption blocking to an 80 kb

plasmid pKC50 from L lactis ssp lactis 57150 Deletion analysis and transposon

mutagenesis indicated the possible involvement of a 100 kDa cell surface antigen A

37 5 kb conjugative plasmid P2520L from L lactis ssp lactis P25 which encodes

adsorption blocking directs synthesis of a unique 30 kDa cell surface antigen (Akcelik

and Tunail, 1992), however, no evidence was provided to support a role for this

protein in phage resistance

The precise nature of the genetic determinants responsible for phage resistance

has not been established in any of the systems described above Despite generating

overlapping subclones of the entire pCI528 genome, Lucey (1992) was unable to

achieve expression of the phage resistance phenotype, suggesting that a large

contiguous fragment or more than one locus was required In E coli strains producing

extracellular polysaccharides (EPS), 17 kb of DNA were required for production and

furthermore, this was divided into three functional loci: the first comprised the genes required for translocation of the EPS across the cell membrane; the second encoded the genes which directed synthesis of the EPS and the third region was suggested to be involved in attachment of the EPS to the cell suface (review by Sutherland, 1993). Thus, it is possible that a complex arrangement of genes is required for EPS production in lactococci also. Nevertheless, cloning and genetic analysis of these determinants would undoubtedly advance the understanding of this phage resistance mechanism.

1.4   RESTRICTION/MODIFICATION (R/M)

Having successfully negotiated adsorption and DNA penetration, phage genomes may encounter restriction endonucleases upon entry to the host cytoplasm. R/M systems were first identified in lactococci by Collins (1956) and several reports have confirmed their widespread distribution (reviewed by Sanders, 1988; Hill, 1993; see Table 1.2). These systems are composed of two separate enzyme activities. An endonuclease, which recognises a specific DNA sequence, cleaves the DNA either within the recognition sequence or at a distance; and a companion methylase which modifies a nucleotide within the same recognition site, thereby protecting the resident DNA from digestion.

The probability that R/M systems in lactococci were likely to be plasmid-encoded was recognised by Limsowtin *et al.* (1978) arising from the observed instability of this phenotype in certain strains. However, it was not until 1981 that Sanders and Klaenhammer provided the first conclusive evidence for a plasmid-located R/M system. The determinants responsible have since been cloned, and localised to a 4.0 kb region of pME100 (Sanders and Shultz, 1990).

The fact that lactococci typically harbour multiple plasmids has often presented difficulties in identifying which plasmids specifically encode the phage resistance

Table 1.2

Lactococcal plasmids which encode R/M

Plasmid	Size(kb)	Source strain		Original Reference
pME100	15 3	LC	KH	Sanders and Klaenhammer (1981)
pIL6	28	LL	IL594	Chopin *et al* (1984)
pIL7	31	LL	IL594	"
pTR2030	46	LL	ME2	Klaenhammer and Sanozky(1985)
pLR1020	30	LC	M12R	Steenson *et al* (1986)
pIL103	5 7	LC	IL964	Gautier and Chopin (1987)
pIL107	15 2	LC	IL964	"
pKR223	36	LL	KR2	Laible *et al* (1987)
pTN20	28	LL	ME2	Higgins *et al* (1988)
pBF61	40	LL	KR5	Froseth *et al* (1988)
pJW563	12	LC	W56	Josephsen and Vogensen (1989)
pJW565	14	LC	W56	"
pJW566	25	LC	W56	"
pFV1001	13	NR		Josephsen and Klaenhammer(1990)
pFV1201	17	NR		"
pTRK12	30 5	LC	TDM1	Sing and Klaenhammer (1991)
pTRK30	28	LC	TDM1	"
pTRK317	15 5	LC	TDM1	"
pAH82	82	LL	DPC220	Harrington and Hill (1992)
pAH90	90	LL	DPC220	"
un-named	131	LL	HID113	Ward *et al* (1992)
p3085-2	15 3	LL	3085	Geis *et al* (1987)
5 un-named	13-18	LC	KH	Vogensen *et al* (1987)
			V32 2	
			T29W5	
			TK5-56	

LC -*L lactis* ssp *cremoris*

LL -*L lactis* ssp *lactis*

NR-not reported

phenotypes observed in particular hosts Two strategies have primarily been employed in the identification of a number of R/M plasmids and indeed other phage resistance plasmids Alterations in the plasmid complement of variants with increased sensitivity to phage was the method by which pIL6 and pIL7 from *L lactis* ssp *lactis* IL964 (Chopin *et al* , 1984), pIL103 and pIL107 from *L lactis* ssp *cremoris* IL594 (Gautier and Chopin, 1987) and pJW563 and pJW566 from *L lactis* ssp *cremoris* W56 (Josephsen and Vogensen, 1989) were recognised The second method of choice involved transfer of candidate plasmids to plasmid-free phage-sensitive hosts where their effect on phage proliferation could be assessed This was accomplished by either conjugal tranfer as with pTR2030 (Klaenhammer and Sanosky, 1985), pKR223 (Laible *et al* , 1987), pBF61 (Froseth *et al* , 1988a) and pTN20 (Higgins *et al* , 1988), or by co-transformation with marker plasmids as occurred with pTRK30, pTRK317 (Sing and Klaenhammer, 1991)

As explained previously, pAH90 was generated following plasmid rearrangement during BIM formation of *L lactis* ssp *lactis* biovar *diacetylactis* DPC220 (Harrington and Hill, 1992) In addition to adsorption inhibition, this cointegrate plasmid also harboured an R/M system which was not active in either component plasmid suggesting that a previously 'silent ' R/M activity was 'turned on' by the comtegration event A second, distinct R/M system was identified on one of the original plasmids, pAH82, which was not active in pAH90

1.4.1 Molecular analyses of lactococcal R/M systems

The determinants responsible for many plasmid-encoded R/M systems have been cloned and include those from pTRK12 and pTRK30 (Sing and Klaenhammer, 1991), pIL103 and pIL7 (Gautier *et al* , 1987), pTR2030 (Hill *et al* , 1989b) and pKR223 (McKay *et al* , 1989) In addition, a number of these systems have been further characterised by DNA sequence analysis

The conjugative plasmid pTR2030 was originally isolated from the prototype phage insensitive strain *L lactis* ssp *lactis* ME2 (Klaenhammer and Sanozky, 1985), and is the most extensively studied lactococcal phage resistance plasmid (see review Klaenhammer *et al* , 1991) pTR2030 encodes both *LlaI* R/M and the AbiA genetic determinants, both of which were cloned on a single 13 8 kb fragment of DNA (Hill *et al* , 1989b) Subcloning and deletion analysis localised the R/M determinants separate from the *abi*A gene Subsequent DNA sequence examination of the methylase gene predicted a protein which contained the 10 and 15 aa consensus sequences common to all type II adenine methylases (Hill *et al* , 1991b) Unusually, both consensus sequences appeared twice, leading the authors to conclude that the protein contained two methylase domains and acted as a covalently-linked dimer modifying the complementary strands of an assymetric recognition site (Klaenhammer *et al* , 1991) Four additional ORF's were found to be located on the same transcriptional unit downstream of the methylase gene, and frame-shift mutations at selected *Eco*RI sites present within these ORF's suggested the involvement of three of these in the expression of the *LlaI* restriction activity (O' Sullivan *et al* , 1995) In addition, a small ORF was identified upstream which exhibited homology to a class of regulatory proteins for type II R/M systems

Although the majority of lactococcal R/M systems studied to date have been plasmid-encoded, this is not always the case Davis *et al* (1993) reported the first isolation of lactococcal methylase determinants from chromosomal sources Two distinct methylases were cloned from the chromosome of *L lactis* ssp *cremoris* UC505 and expressed in *E coli* Selection of clones was based on their ability to fully protect the DNA from cleavage by the cognate *Scr*FI endonuclease first described by Fitzgerald *et al* (1982), however, neither of the clones obtained demonstrated endonuclease activity The amino acid sequences, deduced from the DNA sequences of each of these distinct methylases, contained the highly characteristic motifs of m^5 cytosine methylases (Davis *et al* , 1993; Twomey *et al* , 1993a) The two methylases

reside on adjacent *Eco*RI fragments on the UC505 chromosome and DNA sequence analysis of the intervening region revealed an additional ORF which, according to Twomey *et al* (1993b), is likely to encode the *Scr*FI restriction endonuclease This is not the first instance where two methylase genes have been associated with a single restriction endonuclease In *Streptococcus pneumoniae*, two methylases are associated with the *Dpn*II system, one recognising double-stranded DNA and the other recognising both single- and double-stranded DNA In pneumococci, DNA transformation involves uptake of single-stranded DNA intermediates and thus, the methylation of single-stranded DNA would protect the incorporated DNA against *Dpn*II restriction, while reducing phage infection mediated by dsDNA (Lacks *et al*, 1991) As natural transformation has not been demonstrated in lactococci, this is unlikely to be the explanation for the presence of two methylases in the *Scr*FI system

A third lactococcal R/M system designated *Lla*III, encoded by pJW563 from *L lactis* ssp *lactis* W56 has been cloned (Josephsen and Vogensen, 1989) and sequenced (Nyengaard *et al*, 1993) In this instance, the methylase and restriction endonuclease are encoded by single open reading frames which are divergently transcribed (Nyengaard *et al*, 1993) Although only three lactococcal R/M systems have been analysed to this level, the diversity of genetic organisation is striking

1.4.2 Combinations of R/M systems

R/M systems, in general, do not provide complete resistance but limit phage proliferation by reducing the efficiency of plaquing (EOP) They are inherently leaky, as phage which escape restriction are methylated and consequently immune to restriction in a second round of infection Nevertheless, R/M can be very efficient (EOP 10^{-6}) and provide significant advantages over alternative resistance mechanisms Firstly, the incoming DNA is destroyed (unlike adsorption inhibition where the infecting phage remain in the environment) and secondly, R/M systems contribute to

host survival (unlike Abi where the host dies despite the phage resistance mechanism) Thus, while R/M systems may not be an ideal form of phage resistance in industrial fermentations, their widespread distribution demonstrates their effectiveness in natural ecosystems

In many instances, more than one R/M system has been identified within a single strain pIL6 and pIL7 in *L lactis* ssp *lactis* IL594 (Chopin *et al*, 1984), pIL103 and pIL107 in *L lactis* ssp *cremoris* IL964 (Gautier and Chopin, 1987), pTN20 and pTR2030 in *L lactis* ssp *lactis* ME2 (Higgins *et al*, 1988, Klaenhammer and Sanozky, 1985), pTRK12, pTRK30 and pTRK317 in *L lactis* ssp *cremoris* TDM1 (Sing and Klaenhammer, 1991) and pJW563, pJW565 and pJW566 in *L lactis* ssp *cremoris* W56 (Josephsen and Vogensen, 1989) The additive effect of combined R/M systems was demonstrated by Chopin *et al* (1984) where the EOP of ϕ66 on strains containing both pIL6 and pIL7 was 2×10^{-4} whereas the presence of either plasmid alone gave EOP's of 7×10^{-3} and 8×10^{-2}, respectively Similarly, Gautier and Chopin (1987) showed the additive effect of pIL103 and pIL107 where strains containing both plasmids reduced the EOP of ϕ8 to 10^{-6} whereas the individual reductions provided by each of the plasmids alone were 10^{-2} and 10^{-4}, respectively

Josephsen and Klaenhammer (1990) also assessed the combined effect of multiple R/M systems by constructing strains containing up to three R/M plasmids from different sources Various combinations of pJW563, pFV1001, pFV1201 and pTRK12 were obtained following co-transformation with pVS2 (a marker plasmid which encodes resistance to chloramphenicol and erythromycin) into *L lactis* ssp *lactis* MG1363 No strain was produced containing all four plasmids probably reflecting incompatibility between some of the replicons A reduction in EOP of 10^{-7} was demonstrated for ϕp2 in a strain containing pJW563, pFV1001 and pTRK12 This was higher than the value mediated by any of the plasmids used in isolation, reflecting the efficacy of stacked R/M systems In Section 6, the combined effect of R/M with other phage resistance phenotypes will be discussed

1.4.3 Biochemical analyses of restriction/modification systems

Despite the frequency with which R/M systems have been described in lactococci, few have been investigated at the biochemical level In the majority of cases, host dependent plaquing efficiency was the only evidence provided in support of claims for R/M activity However, Fitzgerald *et al* (1982) described the isolation of a restriction enzyme from *L lactis* ssp *cremoris* UC503 (previously designated *Streptococcus cremoris* F) This type II restriction endonuclease, *Scr*FI, recognised the sequence 5' CC*NGG 3' (where * indicates the point at which the DNA is cleaved) Purification by ammonium sulphate precipitation and successive column chromotography steps permitted a molecular weight determination, by gel filtration, of 34 kDa under non-denaturing conditions (Davis *et al* , 1993) Molecular analysis of this R/M system was described in Section 4 1

Mayo *et al* (1991) identified a restriction endonuclease from *L lactis* ssp *lactis* NCDO 497 designated *Lla*I (unrelated to *Lla*I of pTR2030) This was a typical type II restriction endonuclease which was shown to be an isoschizomer of *Eco*RII, recognising the sequence 5' CC(A/T)GG 3' (cleavage point unknown) The specificities of two R/M systems from two *L lactis* ssp *cremoris* strains have also been determined (Nyengaard *et al* , 1993) *Lla*AI from pFW094 (W9) recognises 5' *GATC 3' while *Lla*BI from pJW563 (W56) recognises 5' C*T(A/G)(C/T)AG 3'

1.5 ABORTIVE INFECTION (ABI)

Abortive infection describes any phage resistance mechanism which interferes in intracellular phage development after the DNA has entered the host without being restricted (Duckworth *et al* , 1981) Therefore, mechanisms which hinder phage

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genome replication, transcription /translation, phage packaging or cell lysis/phage release are all grouped under this term Abortive infections are phenotypically characterised either by the absence of plaques or by reduced EOP's and reduced plaque sizes, which are most probably a reflection of lower numbers of productive infections and limited release of phage progeny (Sing and Klaenhammer, 1990, Durmaz *et al*, 1992, Geis *et al*, 1992) One of the most distinctive aspects of Abi-mediated resistance is that cell survival is low, possibly because corruption of host functions and host DNA degradation have already been initiated prior to operation of the abortive mechanism

pNP40, the first plasmid to be identified as encoding Abi, was isolated by McKay and Baldwin (1984) after conjugal transfer from *L lactis* ssp *lactis* biovar *diacetylactis* DRC3 to *L lactis* ssp *lactis* LM0230 It encoded complete resistance to both prolate and isometric-headed phage as manifested by the absence of plaques on a lawn of cells containing pNP40 Numerous other Abi plasmids have been identified subsequently and are listed in Table 1 3

1.5 1 Molecular analyses of lactococcal *abi* determinants

The genetic determinants for several lactococcal Abi mechanisms have been cloned and four have been analysed at the DNA sequence level *L lactis* ssp *lactis* ME2 contains two *abi* genes which are located on two different plasmids pTR2030 and pTN20 Analysis of the mechanisms by which these genes mediate abortive infection are described in Section 5 2 The *abi* gene of pTR2030 was the first to be analysed to sequence level This plasmid was initially isolated following its co-transfer from ME2 to *L lactis* ssp *lactis* LM0230 together with the Lac plasmid, pTR1040 (Klaenhammer and Sanozsky, 1985) pTN20 was transferred by a similar process into *L lactis* ssp *lactis* LM2301, although it was not immediately recognised as encoding Abi (Higgins *et al*, 1988) The *abi* genes from both plasmids have been cloned (Hill *et al*, 1989b) and sequenced (Hill *et al*, 1990b, Durmaz *et al*, 1992) *abiA* (formerly *hsp*) from

Table 1.3
Lactococcal plasmids which encode abortive infection

Plasmid	Size(kb)	Source		Cloned/ Sequenced	Add. ø Resistances	tra	Original Reference
pNP40	64	LD	DRC3	-/-	___	+	McKay and Baldwin (1984)
pTR2030	48	LL	ME2	+/+	R/M	+	Klaenhammer and Sanozky (1985)
pCI750	65	LC	UC653	+/-	___	+	Baumgartner et al (1986)
pIL105	8 7	LC	IL964	+/-	___	-	Gautier and Chopin (1987)
pKR223	38	LD	KR2	+/-	R/M	+	Laible et al (1987)
pTN20	28	LL	N1	+/+	R/M	+	Higgins et al (1988)
pCI528	46	LC	UC503	-/-	Ads	+	Costello (1988)
pAJ1106	106	LD	4942	-/-	___	+	Jarvis (1988)
pCLP51R	90	LL	33-4	-/-	___		Dunny et al (1988)
pBF61	42	LL	KR5	+/-	R/M	+	Froseth et al (1988)
pCI829	44	LL	UC811	+/+	___	+	Coffey et al (1989)
pNP2	134	LL	WW4	-/-	___	+	Steele et al (1989)
pCC34	34	LC	C3	-/-	___	+	Murphy et al (1988)
pEB56	56	LC	EB7	-/-	___	+	"
pJS88	88	LL	11007	-/-	___	+	"
pJS40							"
pIL416		LL	IL416	+/+	___		Cluzel et al (1991)
pBU1-8	64	LD	BU1	-/-	___	-	Geis et al (1992)
unnamed	131	LL	HID113	-/-	R/M	+	Ward et al (1992)

LL -*L lactis* ssp *lactis*, LC -*L lactis* ssp *cremoris*, LD -*L lactis* ssp *lactis* biovar *diacetylactis*

pTR2030 is 1887 bp in length and encodes a protein with a predicted molecular mass of 73 8 kDa It is preceded by a constitutively expressed promotor (Hill *et al* , 1990b) Sequence data for *abiC* (pTN20) revealed a gene 1056 bp in length which shares no homology with *abiA* (Durmaz *et al* , 1992) Both pTR2030 and pTN20 encode R/M activities in addition to Abi (Hill *et al* 1989a, Durmaz *et al* , 1992)

pCI829 is a conjugative plasmid isolated from *L lactis* ssp *lactis* UC811 following co-transfer with a Lac plasmid (Coffey *et al* , 1989) Genetic analysis of the *abi* determinant from pCI829 (Coffey *et al* , 1991) revealed a sequence identical to *abiA* from pTR2030 despite evidence by Hill *et al* (1990b) that the *abiA* sequence was not widely distributed in lactococci This is the only instance in which independently isolated Abi systems have been found to be identical

A fourth *abi* gene, *abi416* (*abiB*), has also been analysed to the sequence level (Cluzel *et al* , 1991) This gene was cloned from total cell DNA isolated from *L lactis* ssp *lactis* 416 and analysis of phage resistant clones in *L lactis* ssp *lactis* IL1403 revealed a recombinant plasmid, pIL416, which contained a 753 bp open reading frame which mediated resistance to øbIL66 The gene was preceded by an ISS1 element which was shown to contain a putative gram positive promotor sequence which was responsible for expression of the Abi phenotype

1 5.2 Mode of action of lactococcal *abi* genes

The molecular basis of abortive infections in lactococci is not well understood but recent investigations have provided some insights The effect of Abi on lactococcal phage DNA replication was first investigated by Hill *et al* (1991a) when total DNA was isolated from ø31-infected *L lactis* NCK203 cells with and without pTR2030 (AbiA) The increase in intracellular phage DNA concentration over time in plasmid-free cells reflected normal øDNA replication No such increase was observed in cells containing pTR2030 demonstrating that the presence of this Abi mechanism blocked

phage DNA replication, although the original phage genome could be detected in the host cytoplasm Analyses of phages related to ø31, which were detected during prolonged use of pTR2030-containing transconjugants in commercial fermentations, demonstrated that phages sharing a similar origin of genome replication to ø31 were inhibited by the Abi mechanism while phages with unrelated *ori's* were unaffected (Hill *et al* , 1991b)

The Abi mechanisms encoded by pBU1-8 (Geis *et al* , 1992)and pTN20 (AbiC) have been shown to differ from that encoded by pTR2030 in that phage DNA replication did take place, although, in the case of pBU1-8, the rate of replication was considerably reduced Intracellular øP008 DNA concentration increased slowly over a five hour period following infection of a pBU1-8-containing host whereas only sixty minutes were required before high phage DNA concentrations were obtained within a sensitive host (Geis *et al* , 1992) In the case of AbiC (Durmaz *et al* , 1992), øp2 DNA replication was not visibly inhibited

Further attempts were made to elucidate the mechanisms involved in abortive infection as encoded by *abiC* and pBU1-8 No transcription of phage DNA was detected in øP008-infected cells containing pBU1-8 by Geis *et al* (1992) suggesting a block at this stage Using monoclonal antibodies directed against the capsid protein of the small isometric phage øu136, Moineau *et al* (1992) established that production of this protein was reduced by 50% in cells containing AbiC, indicating that the target for the mechanism possibly involved the phage transcription, translation or packaging processes These results confirm that not all Abi systems operate on a single target and that the term describes a variety of late-acting phage resistance mechanisms This highlights the potential for exploitation of multiple Abi systems to inhibit phage at sequential steps in the lytic cycle in order to provide higher levels of phage resistance

Many Abi systems have been identified in gram negative bacteria In the case of two of these systems, in particular, the mechanisms have been studied in great detail and the molecular basis has been elucidated In *Vibrio cholerae* biotype El Tor cells,

production of stable concatemeric ⌀149 DNA intermediates was prevented due to their failure to associate with the cell membrane The resulting concatemeric DNA was unsuitable for subsequent cleavage into monomers and packaging into phage heads In addition, synthesis of late proteins was reduced and transient (Chowdury *et al* , 1989) A 1 2 kb fragment of DNA encoding 14 kDa and 22 kDa proteins was found to be sufficient to confer resistance The 14 kDa protein was highly hydrophobic in nature and inserted itself into the cell membrane and thus, destabilised the concatemeric DNA intermediates by hindering their binding to the cell membrane (Biswas *et al* , 1992)

F plasmid-containing *E coli* cells abort phage T7 infections (Kruger and Bickle, 1987) In addition, a number of T3/T7 recombinant phages were assessed for their ability to plaque on F+ cells All recombinant phages in which gene 1 was derived from phage T7 were excluded Gene 1 from phage T7 terminates at an ochre stop codon and exclusion arose from suppression of this stop codon, by ribosomal misreading, resulting in a considerably extended protein The authors concluded that abortivity encoded by the F plasmid resulted from production of junk proteins

1.6   COMBINATIONS OF PHAGE RESISTANCE MECHANISMS

Numerous phage insensitive strains have been described which harbour more than one phage resistance determinant (Table 1 4) Either two phage resistance plasmids may be resident in a single strain, e g *L lactis* ssp *cremoris* IL964 (Gautier and Chopin, 1987), or a single plasmid may encode more than one phage resistance gene, e g pTR2030 (Hill *et al* , 1989a) The benefits to a host which contains multiple phage resistances are two-fold either the level of resistance to a particular phage can be increased, and/or the range of phage to which it is resistant, may be extended The efficacy of combining R/M systems was discussed previously, when it was shown that elevated phage resistance was observed when more than one R/M plasmid was present

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Table 1.4

Naturally occuring strains which possess more than one phage resistance determinant

Strain	ø^r plasmids	Ads. Inh.	R/M	Abi	Reference
LL ME2	pME0030	+			Klaenhammer *et al* (1991)
	pTN20		+	+	
	pTR2030		+	+	
LL IL594	pIL6		+		Chopin *et al* (1984)
	pIL7		+		
LC IL964	pIL103		+		Gautier and Chopin (1987)
	pIL105			+	
	pIL107		+		
LC W56	pJW563		+		Josephsen and Vogensen (1989)
	pJW565		+		
	pJW566		+		
LC TDM1	pTRK12		+		Sing and Klaenhammer (1991)
	pTRK30		+		
	pTRK317		+		
LL KR2	pKR223		+	+	Laible *et al* (1987)
LC UC503	pCI528	+		+	Costello (1988)
LL KR5	pBF61		+	+	Froseth *et al* (1988)
LL HID113	pHID113		+	+	Ward *et al* (1992)
LD DPC721	pAH90	+	+		Harrington and Hill (1992)

LL -*L lactis* ssp *lactis*, LC -*L lactis* ssp *cremoris*, LD -*L lactis* ssp *lactis* biovar *diacetylactis*

in a host, e g pIL6 and pIL7 in IL594 (Chopin *et al* ,1984) and pIL103 and pIL107 in IL964 (Gautier and Chopin, 1987)

Several instances have been recorded where determinants for more than one phage resistance determinant have been located on a single plasmid The most frequently observed combination has been R/M and abortive infection The effectiveness of this combination may be attributed to the survival value conferred on the cell by the early mechanism, R/M, and the reduced phage proliferation mediated by the late mechanism in those instances in which phage escape restriction, abortive infection In this context, the 'survival value' refers to the probability of a cell surviving the phage attack

Of course, it can be difficult to determine the presence of two phage resistance systems in a single strain since the effect of one can be masked by the other resistance The low level of overall resistance mediated by pBF61 (in that plaques could still be detected) permitted the immediate recognition that it encoded both R/M and Abi (Froseth *et al* , 1988a) Phage propagated on hosts containing pBF61 plaqued with an increased EOP in a second round of plaquing but retained the reduced plaque size and a modest reduction in EOP On the contrary, the multiple resistances encoded by pTR2030, pTN20 and pKR223 were not immediately recognised The use of the AbiA-resistant ∅48, a phage isolated from whey samples following the extended use of pTR2030-containing transconjugants in industrial fermentations, revealed the presence of the *Lla*I system on pTR2030 ∅31, the phage previously employed, although inhibited by both R/M and Abi, did not permit distinction between them since no plaques were recovered on strains containing pTR2030 (Hill *et al* , 1989b) In the case of pKR223, an R/M system, which was not active against the prolate-headed ∅c2 used initially in characterising the Abi phenotype, (McKay *et al* , 1989) was identified when the small isometric-headed ∅sk1 was used in the investigation of the phage resistance it encoded It was during the subcloning of the R/M determinants on pTN20, a previously undetected *abi* gene was localised (Durmaz *et al* , 1992) Combination of

phage resistance mechanisms in these instances conferred an increase either in the degree of phage resistance, e g pTR2030 and pBF61, or in the range of phage to which the host was insensitive, e g pTR2030 and pKR223 Thus, it may be possible that many of the phage resistance plasmids currently under investigation encode additional mechanisms which are either not active against the phage used in the study or which are obscured by other mechanisms Localisation and subcloning of phage resistance determinants and the use of a variety of phage types in assessing the nature of resistance conferred should ensure a more complete understanding of the phage resistances encoded by individual plasmids

The association of early and late mechanisms includes the combination of adsorption inhibition with abortive infection This combination has been recorded in the case of pCI528 (Costello, 1988) which mediates resistance to phage in L $lactis$ ssp $cremoris$ UC503 by abortive infection and in the case of øc2, by adsorption inhibition It is not known whether the inhibition of this phage was due solely to adsorption blocking or if the abortive infection mechanism contributed to the resistance observed When transferred to the L $lactis$ ssp $lactis$ MG1363, however, pCI528 conferred resistance to øc2 and three other phage by adsorption inhibition alone

A unique combination of phage resistance mechanisms was described in the case of the cointegrate plasmid pAH90 which mediated resistance to øc2 by both R/M and adsorption inhibition in the laboratory strain L $lactis$ ssp $lactis$ MG1614 However, the adsorption blocking mechanism was not active against øD1 in the parent strain L $lactis$ ssp $lactis$ biovar $diacetylactis$ DPC721

pNP40 encodes two abortive infection determinants (Chapters 3 and 4, this thesis) which together confer complete resistance to ø712 in the laboratory strain L $lactis$ ssp $lactis$ MG1614 Although phage proliferation is eliminated, cell survival is comparatively low due to the absence of an early mechanism operating against this phage However, pNP40 does encode a DNA penetration blocking mechanism active against the prolate-headed phage øc2 (Chapter 5, this thesis) Thus, pNP40 is the only

plasmid identified to date which encodes three independent phage resistance mechanisms

A total of five phage resistance determinants on three plasmids were identified in *L lactis* ssp *lactis* ME2, the prototype phage resistant strain (Klaenhammer *et al*, 1991) It contains representatives from all three phage resistance categories pME0030 encodes adsorption inhibition while pTR2030 and pTN20 each encode both R/M and abortive infection determinants Molecular analysis of some of these genes is at an advanced stage with sequence data available for both *abi* genes and for the pTR2030 *LlaI* R/M system The abortive infection mechanisms have been shown to act at consecutive stages in the phage lytic cycle, namely inhibiting phage DNA replication and transcription/translation, thereby minimising phage proliferation Thus, the phage lytic cycle on host ME2 may be interrupted at four different stages the adsorption process may be blocked, intracellularly, the phage DNA may be subject to attack by the action of two independent restriction endonucleases, phage DNA replication may be inhibited or phage protein synthesis may be disrupted

As production of strains with enhanced phage resistance properties is a goal in the generation of improved starter cultures, several attempts have been made to introduce phage resistance plasmids from diverse sources into single strains in an effort to assess their unified effects on phage proliferation Coffey *et al* (1989) introduced pCI829 from *L lactis* ssp *lactis* UC811 and pCI750 from *L lactis* ssp *cremoris* UC653 into MG1363 by conjugation to study the effectiveness of their combined Abi mechanisms Complete resistance to øc2 was obtained when both plasmids were present in the host whereas only partial resistance was observed when either plasmid was assessed alone The additive effect of three R/M plasmids co-transformed into the strain *L lactis* ssp *lactis* MG1363 (Josephsen and Klaenhammer, 1990) was discussed previously and resulted in a reduction in the EOP for øp2 of 10^{-7}

Sing and Klaenhammer (1991) conducted a study to assess the effect of combining R/M plasmids with the abortive infection mechanism (but not the R/M

system) of pTR2030 in *L lactis* ssp *lactis* LM0230 Resistance could be assessed for øc2 and øp2 only, as the R/M system on pTR2030 was not active against these two phages Strains carrying both pTR2030(AbiA) and pTRK30(R/M) gave a level of resistance against øp2 stronger than was conferred by any other system previously investigated by these researchers They found that this plasmid combination further reduced infective centre formation compared to that conferred by pTRK30 alone and reduced cell death compared to pTR2030 alone Phage øc2 plaqued with a reduced EOP relative to strains carrying either plasmid alone and the plaque size was also considerably reduced reflecting the operation of both the R/M and Abi mechanisms

These studies attested to the usefulness of conjugation and transformation techniques in the construction of strains with enhanced phage resistance properties, however, pTRK12(R/M) had a negative effect on the expression of the AbiA phenotype (see Section 9) with no reduction in the plaque size of øc2 on strains containing both pTR2030 and pTRK12 and only the R/M phenotype was observed This demonstrates that this type of strategy, although potentially very effective, is not universally applicable

1.7 INTRODUCTION OF PHAGE RESISTANCE PLASMIDS INTO COMMERCIAL STRAINS

Following the discovery that the phage resistance plasmid pNP40 was conjugative, McKay and Baldwin (1984) proposed a conjugal strategy for the construction of food-grade bacteriophage resistant strains for the dairy industry This approach was subsequently employed by Sanders *et al* (1986) in the construction of derivatives of commercial starter cultures containing pTR2030 and proved to be the first example in which the genetic manipulation of strains yielded a practical application The novel strains have improved phage resistance characteristics and have been exploited

successfully under industrial conditions This approach has also been used successfully by Kelly *et al* (1990) and Harrington and Hill (1991) in the introduction of pCI750 and pCI528, and pNP40, respectively into commercial starter strains

The advantage of this strategy is that it is non-recombinant and exploits a method of gene transfer used naturally by lactococci, thus yielding food-grade starters All of these genetically modified strains have been used successfully in industry but, in the case of pTR2030 transconjugants, prolonged use under industrial conditions resulted in the detection of several phage which were shown to be insensitive to the pTR2030-encoded R/M and Abi phage resistance mechanisms One such phage, ø50, was found by Hill *et al* (1991b) to have protected itself against pTR2030 by effectively cloning an active portion of the *Lla*I methylase determinant This enabled the phage to self-modify its DNA and remain insensitive to the action of *Lla*I In addition to being insensitive to the *Lla*I R/M system, ø50 demonstrated insensitivity to AbiA due to the absence of the specific *ori* which is the target of action for this mechanism These methods of counterdefence exemplify the ability of phage to adapt in the dairy plant to different types of newly introduced resistance mechanisms

More recently another phage, øul37, has evolved in response to pressure exerted by the activity of the AbiC mechanism on pTRK99 (Moineau *et al* , 1994) Restriction mapping indicated that this phage was a derivative of øul36 (an AbiC sensitive phage) which had acquired a large DNA fragment from the chromosome of the resistant transconjugants Despite extensive homology at the genomic level, øul37 differed considerably from øul36 having a longer tail, a different base plate and a different origin of replication The DNA acquisition by øul37 was specific and reproducible in that all mutant phages isolated contained the same discreet fragment of DNA When the chromosomal DNA implicated in the formation of øul37 was interrupted by site-specific integration, the re-emergence of øul37 during subsequent infections with øul36 was prevented This example shows the potential of the host to act as a reservoir of new genetic material for phage evolution in industrial fermentations

and also demonstrated how molecular technology can be used not only to establish the origin of newly acquired DNA but also to prevent the re-occurrence of the recombination event

1.8 NOVEL PHAGE RESISTANCE MECHANISMS

In recent years, an increased knowledge of the molecular biology of lactococcal phages has permitted the development of phage resistant strains by two novel methods Hill *et al* (1990a) exploited bacteriophage DNA as a source of new phage resistance determinants When a 1 4 kb DNA fragment from ø50 (a phage resistant to pTR2030 which was isolated from the factory environment following prolonged use of pTR2030-containing transconjugants) was introduced into *L lactis* ssp *lactis* LM0230, infection by ø50 resulted in a reduced EOP and plaques which were smaller in size than on the original host This phenotype, which is reminiscent of Abi-like mechanisms, was designated *per* (phage encoded resistance) Sequence analysis revealed a 500 bp region rich in secondary structure which contained the ø50 origin of replication According to Hill *et al* (1991a) the presence of the *per* locus inhibited ø50 DNA replication by competition for essential phage replication factors Furthermore, proliferation of phages sharing homology with the ø50 *ori*, e g ø48, were also inhibited by *per* whereas phage with unrelated *ori*'s, e g ø31, were unaffected Thus, a universally applicable method of generating resistant strains was devised which was specific for phages of a group sharing similar *ori's* O'Sullivan *et al* (1993) confirmed this by generating a strain resistant to ø31 by cloning of the ø31 origin of replication

An alternative strategy for generating phage resistant strains was devised by Kim and Batt (1991) and involved the use of antisense mRNA Cloning of an ORF gp51C (function unknown) from the *L lactis* ssp *cremoris* øF4-1, in the antisense orientation relative to a plasmid-encoded promoter, resulted in a >99% decrease in EOP

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and reduced the plaque size of øF4-1 Although this method was previously employed in the regulation of bacterial and plant genes, the mechanism by which antisense mRNA operates is not fully understood One possible explanation is that it causes the production of a non-translatable double-stranded RNA hybrid When only a portion of the ORF was used in the antisense orientation or the entire ORF in the sense orientation, bacteriophage proliferation was not inhibited

gp18C and gp24C are two additional ORFs from øF4-1 both of which are believed to be essential for phage proliferation and which have been shown to be highly conserved among a range of phages, however, antisense RNA directed against them reduced the EOP of phages carrying these sequences by a factor of only 0 5 (Kim *et al* , 1992) Subsequently, Chung *et al* (1992) cloned the major capsid protein (MCP) of øF4-1 in the antisense orientation which resulted in an equally limited reduction in EOP These data suggest that the usefulness of this method is best confined to situations involving ORFs with a low level of transcription/translation since antisense mRNA directed against these appears to be more effective than when it is directed against genes which are transcribed in relatively high amounts The use of antisense RNA as a method of devising phage resistant strains is also limited to phage which have been analysed at the DNA sequence level as DNA sequence data is required to identify appropriate ORF's

1.9 FACTORS INFLUENCING PHENOTYPIC EXPRESSION OF PHAGE RESISTANCE GENES

A number of factors have been shown to influence the phenotypic expression of phage resistance genes, e g phage type, host strain, temperature of infection, gene copy number and the presence of additional DNA such as conjugal elements This section discusses the level of expression of a number of phage resistance genes under different circumstances

It has been observed that genes which confer insensitivity to one phage may be completely ineffective against another For example, pKR223 encodes two phage resistance mechanisms an Abi system which is active only against the prolate-headed phage øc2, and an R/M system active only against the small isometric-headed ø712 (Laible *et al* , 1987) Alternatively, a single determinant may confer variable degrees of resistance to different phages, e g *abiA* (pCI829) completely inhibits ø712 while conferring only partial resistance to the prolate-headed øc2 (Coffey *et al* , 1989)

In general, small isometric-headed phages are more susceptible to R/M systems than prolate-headed phages Their larger genome size (approx 30 kb vs 20 kb) and the consequent increased probability of their containing suitable recognition sites has been proposed as a possible explanation Variation has also been recorded in phage responses to Abi systems Two phages, ø48 and ø50, possess origins of replication which render them unsusceptible to the AbiA mechanism which appears to target ø31-type replication origins (Alatosova *et al* , 1991)

Phage resistance has also been shown to be strain dependent Jarvis and Klaenhammer (1986) introduced pTR2030 by conjugation into several *L lactis* ssp *lactis* and *cremoris* strains and examined its effect on the proliferation of their homologous phages Phage øc2, the only phage examined which was homologous for more than one of these strains, was completely inhibited in *L lactis* ssp *cremoris* KHA2 while only partial resistance was observed in *L lactis* ssp *lactis* LM0230 background, indicating a strain dependent alteration in phage resistance pCI528 exhibited an Abi-type resistance to four phages in its parent strain UC503 and, in the case of øc2, adsorption inhibition was also observed However, in the laboratory strain *L lactis* ssp *lactis* MG1363, all four phages examined were inhibited solely by adsorption blocking and Abi involvement in the observed phenotype was ruled out (Costello, 1988) This may reflect the absence of a phage homologous for MG1363 against which the Abi system is active rather than the failure of the Abi determinant to be expressed in this host Similarly, the adsorption blocking phenotype of pAH90 was

not detected in its native strain *L lactis* ssp *lactis* biovar *diacetylactis* DPC721 but was identified against øc2 in the laboratory strain MG1614 (Harrington and Hill, 1992)

Gene copy number has also been shown to have a direct effect on phage resistance Casey *et al* (1992) was the first to make this observation following introduction of a single copy of *abiA* from pCI829 into the *L lactis* ssp *lactis* CH919 chromosome A decrease in the level of resistance to both phages ø712 and øc2 was recorded relative to the plasmid-encoded *abiA* Amplification of the integrated DNA sequences within the chromosome resulted in an accompanying increase in the level of phage resistance Analysis of the intracellular DNA content of infected cells containing *abiA* in single copy versus multiple copies indicated that the copy number of the *abiA* gene present in a strain directly affected the degree of phage DNA replication permitted and, consequently, the level of insensitivity exhibited by the host Dinsmore *et al* (1994) confirmed that gene copy number has an effect on expression of *abiA* (pTR2030) by comparing its effectiveness following its introduction into hosts on low and high copy number vectors or as a single copy by chromosomal integration The site of chromosomal integration was also shown to have an effect on expression levels This was attributed to activity of adjacent promoters as elevated levels of mRNA were detected when integration occurred in specific sites

O'Sullivan *et al* (1993) observed a direct copy number effect when they cloned the *per* locus from ø50 into two lactococcal cloning vectors differing in copy number *Per* cloned on the low copy number vector had a negligible effect on phage proliferation whereas when cloned on a high copy number replicon, *per* reduced the EOP of ø50 considerably

Romero and Klaenhammer (1990) reported that recombination between the genes encoding the R/M and Abi activities of pTR2030 with conjugal elements in lactococci resulted in enhanced phage resistance Mobilisation of pTK6, a recombinant plasmid containing the pTR2030 DNA sequences responsible for R/M and Abi, resulted in the generation of the novel plasmids pTRK78 and pTRK79, which

contained a conjugal element in addition to pTK6 DNA Additional reductions of 10^{-2}

to 10^{-5} were observed in the efficiencies of plaquing of phages ϕc2 and ϕ48 The

authors concluded that the enhanced phage resistance was a direct consequence of the

physical interaction between pTR2030-derived sequences and a conjugal element

resident in the donor strain It was suggested that the presence of an ISS1-type element

on pTK6 was necessary for this recombination In addition to pTR2030, IS elements

are associated with many other phage resistance plasmids, e g pCI829, pIL416,

pCI750 and pNP40, and in one instance, have been implicated as having a role in gene

expression On pIL416, expression of *abiB* (formerly *abi416*) is actually mediated by

promotor sequences within an adjacent ISS1 (Cluzel *et al* , 1991)

Distal sequences may also play a role in regulation of expression of the *abi* gene

of pKR223 Although it has been localised, the *abi* determinant could not be physically

separated from the R/M activities suggesting that sequences upstream of the R/M

system were required for expression of the Abi phenotype

While IS elements have often been implicated in plasmid rearrangements, they

were not evident in the formation of the cointegrate phage resistance plasmid pAH90

Cointegration of pAH82 and pAH33 was a precise event without the duplication of

DNA fragments typically associated with IS-mediated recombination events However,

cointegration resulted in the activation of an adsorption blocking mechanism and an

R/M system The precise molecular basis of the alteration in phenotypic expression is

unknown (Harrington and Hill, 1992)

Suppression of a phage resistance phenotype has been recorded on two

occasions as a direct consequence of the introduction of additional plasmid DNA Sing

and Klaenhammer (1991) reported that the reduced plaque size characteristic of the

AbiA phenotype of pTR2030 was not observed against ϕc2 when the R/M plasmid

pTRK12 was co-resident, although alternative R/M plasmids could be introduced into

pTR2030 transconjugants which resulted in additive resistance effects (Sing and

Klaenhammer, 1991, Josephsen and Klaenhammer, 1990) The authors suggested that

'incompatibility of gene expression' was responsible for this effect In similar circumstances, Higgins *et al* (1988) observed that the R/M activities of pTN20 were suppressed when the Lac plasmid pTR1040 was present in the strain

Thus, while phage resistance may be conferred by a single gene, its expression may be controlled by many other factors including additional ORFs, IS elements, regulatory DNA sequences and the proximity of conjugal elements, which may enhance or reduce its effect on phage proliferation

1.10 CONCLUSIONS

The facility with which phage can overcome resistance mechanisms suggests that they will continue to disrupt dairy fermentations in years to come and that constant vigilance will be required to achieve effective control of phage proliferation in cheese plants The success of transconjugants containing selected phage resistance plasmids, e g pTR2030, has depended on their multi-mechanism nature Notwithstanding this, new phage have arisen which are neither susceptible to the R/M nor the Abi mechanisms of pTR2030

This provides a compelling argument for the necessity of continuing research into new phage resistance mechanisms The identification and characterisation of unrelated systems should identify a broad range of native lactococcal plasmids which can be introduced into starter cultures In depth knowledge of their mechanisms permits the identification and combination of plasmids encoding complementary phage resistance systems, further extending their usefulness A strategy for rotation of different bacteriophage resistances in a single-strain starter culture system has been developed by Sing and Klaenhammer (1993) whereby the plasmids pTR2030 (R+/M+ and AbiA+), pTN20 (AbiC+ R+/M+), pTRK11 (R+/M+) and pTRK68 (R+/M+) were rotated in the commercial strain NCK203 This strategy was found to be effective in

particular if the pTR2030 transconjugant was used when high phage numbers were encountered

The advantage of this type of applied research is the immediacy with which it can be implemented as whole native plasmids are introduced using non-recombinant food-grade genetic technology However, plasmid incompatibility and incompatibility of gene expression may result from the introduction of large quantities of unnecessary or cryptic DNA (phage resistance is often encoded on large plasmids) This could adversely affect the fermentative capabilities of the strain

The development of well-characterised food-grade vectors could facilitate the introduction of genetically engineered phage resistance genes into strains with minimal disruption, as less cryptic DNA would be introduced Froseth and McKay (1991a) have developed a food-grade vector using nisin as a selective marker This vector has been used to clone the phage resistance gene from the Abi plasmid pBF61 and could be used as a prototype 'genetically-engineered food-grade' starter in food trials

The use of genetic engineering would permit control and enhancement of the expression of phage resistance genes by the introduction of additional DNA, e g conjugal elements, regulatory DNA sequences and control ORF's which have been shown to maximise gene expression It would also enable the introduction of novel phage resistance mechanisms, e g cloned *ori's* and cloned antisense RNA in the control of phage which are intractable to existing phage resistance mechanisms Furthermore, these methods are less susceptible to phage counterdefense by single point mutation

The most exciting discoveries in this dynamic field of research have been the innovative ways in which phages evolve to counteract resistance mechanisms This will continue to challenge the dairy industry and guarantee that constant monitoring for new phage and adjustment of starter programs will remain a feature of dairy fermentations

CHAPTER II

MATERIALS AND METHODS

2.1 Bacterial strains, plasmids and culture conditions. Strains, plasmids and phages used in this study are listed in Table 2 1 Lactococcal cultures were grown at 30°C in M17 media supplemented with 0 5% glucose or lactose as appropriate (Terzaghi and Sandine, 1975) *Escherichia coli* cultures were propagated in Luria-Bertani broth and incubated at 37°C (Sambrook *et al*, 1989) M13 phage was propagated as outlined by Sambrook *et al* (1989) pAM401 (Wirth *et al*, 1986) and various derivatives were maintained in lactococci using chloramphenicol at 10 µg/ml and in *E coli* using tetracycline at 10 µg/ml and/or chloramphenicol at 20 µg/ml

2.2 Plasmid and phage DNA preparation. The lysis procedure of Anderson and McKay (1983) was used to isolate plasmid DNA from lactococcal strains *E coli* plasmid DNA was obtained according to the method of Birnboim and Doly (1979), and large volumes were purified by caesium chloride-ethidium bromide density gradient ultracentrifugation in a Beckman VT165 rotor Lactococcal phage DNA was isolated by the method of Fitzgerald *et al* (1982) with the modifications described by Coveney *et al* (1987)

2.3 Restriction endonucleases and molecular cloning techniques. Restriction enzymes, the Klenow fragment of DNA polymerase I and T4 DNA ligase were obtained from Boehringer Corp Dublin, Ireland DNA digestions and ligations were performed as outlined by Sambrook *et al* (1989) DNA fragments were isolated from agarose gels using the Gene Clean Kit II (BIO 101, La Jolla, CA)

2 4 Electroporation of bacteria. Electroporation of lactococcal strains was executed according to the procedure of Holo and Nes (1989) with the Bio-Rad Gene Pulser apparatus (Bio-Rad Corp, Richmond, CA) *E coli* transformations were performed using the conditions outlined in the Bio-Rad manual

Table 2 1

Bacterial strains and phages

	Relevant characteristics	Reference
Strains		
E coli		
HB101	Transformation host for pAM401 derivatives	Boyer & Roulland Dusoix (1969
TG1	Transformation host for M13 derivatives	Yannish-Peron *et al* (1985)
L lactis ssp *lactis*		
MG1614	Plasmid-free derivative of 712, homologous host for øc2 and ø712	Gasson (1983)
MG1614/pNP40	MG1614 transconjugant containing pNP40	DPC* culture collection
PG001	MG1614 transconjugant containing pPG01	Chapter 3 this thesis
PG020	MG1614 transconjugant containing pCG1	Chapter 4, this thesis
PG023	MG1614 transconjugant containing pPG23	Chapter 4 this thesis
VEL1122	RecA deficient derivative of MG1363	Duwat & Gruss (1994)
UC317	Wild-type strain	UCC culture collection
UC653	Wild-type strain	UCC culture collection
HO2	Wild-type strain	UCC culture collection
C2	Wild-type strain	UCC culture collection
712	Wild type strain	UCC culture collection
ML3	Wild-type strain	UCC culture collection
952	Wild-type strain	UCC culture collection
biovar *diacetylactis*		
DRC3	pNP40 parent strain, homologous host for ødrc3	UCC culture collection
18-16	alternative host for ødrc3	UCC culture collection
L lactis ssp *cremoris*		
UC653	Wild-type strain	UCC culture collection
Phages		
L lactis ssp *lactis*		
øc2	prolate-headed phage for MG1614	UCC culture collection
ø712	small isometric headed phage for MG1614	UCC culture collection
ødrc3	homologous phage for DRC3 and 18-16	UCC culture collection
E coli		
M13mp18/19		Yannish-Peron *et al* (1985)

* Dairy Products Centre, Moorepark, Fermoy, Co Cork

2.5 Nucleotide sequence analysis. Relevant DNA fragments were cloned in M13mp18 and M13mp19 vectors (Yannish-Perron *et al*, 1985) The nucleotide sequence was determined using both single-stranded M13 and alkali-denatured pPG01 and pCG1 templates and the Sequenase 2 0 Kit (US Biochemical, Cleveland, OH) or the Taq Track sequencing system (Promega Corp, Madison, WI) Sequencing was initiated using commercial M13 primers and continued with specific synthetic 17-mer primers prepared using a DNA synthesiser (PCR-MATE, Applied Biosystems, Foster City, CA) Each strand of DNA was sequenced at least once Sequencing gels were run as outlined by Bio-Rad Sequence data were analysed by the Gene Jockey and DNAstar database software programmes (Apple Computers Inc, Cupertino, CA)

2.6 DNA hybridisation. DNA was transferred from 0 7% agarose gels to Hybond-N+ nylon membrane (Amersham, UK) by capillary blotting DNA probe fragments were isolated from agarose gels using the Gene Clean Kit II (BIO 101, La Jolla, CA) and homologous DNA was detected by the ECL gene detection system (Amersham, UK)

2 7 Detection of *recA* genes in lactococcal strains using PCR. Lactococcal cultures were grown in M17 supplemented with 0 5% glucose or lactose as appropriate for 14-16 h 1 5 ml of culture was centrifuged at 15,000 rpm for 5 min and resuspended in Ringers solution (Merck, Darmstadt, Germany) before the centrifugation was repeated The cells were finally resuspended in one ml of Ringers solution and subjected to lysis using the 'shake-it-baby' cell disrupter (Biospec Products, Bartleville, OK) for 7 min in the presence of glass beads (106 microns, Sigma Corp, Poole, UK) Glass beads were sedimented using centrifugation and 5 ml of supernatant was used as template in the PCR reaction 17-mer primers were chosen which were specific for the chromosomal $recA_L$ gene (forward primer - 5'

CGTGATAAAGCATTGGC 3', reverse primer -5' AAAGCTGTAGTTTCTTC 3')
and the pNP40-encoded $recA_{LP}$ gene (forward primer -5' TTAGCTATTCTCAAAGC
3', reverse primer - 5' ACTCCAAGTTGAAGTGC 3') Reactions were performed
using the Promega *Taq* polymerase system according to the manufacturers instructions
The Hybaid Omnigene PCR system was programmed as follows template DNA was
denatured for 4 min at 94°C followed by 30 cycles of [(94°C x 1min) + (50°C x 1min)
+ (72°C x 1min)]

2.8   Phage assays. Adsorption of phage to host cells was measured by adding 0 7
ml of a late log-phase culture and 50 μl of calcium chloride to 0 7 ml of phage (10^5
pfu/ml) Following incubation for 15 min at room temperature, the phage-host mixture
was centrifuged for 10 min and the supernatant assayed for phage   Percentage
adsorption was calculated as 100 x [control titre-residual titre][control titre]$^{-1}$

The efficiency of centre of infection (ECOI) was measured using the method of
Sing and Klaenhammer (1990) Cells were infected with phage at a multiplicity of
infection (moi) of 0 1 and incubated for 10 min to permit phage to adsorb to hosts
Efficiency of centre of infection was calculated as [pfu of the infected resistant
host][pfu of the infected sensitive host]$^{-1}$

Cell survival was assayed as described by Behnke and Malke (1978) Surviving
cells were enumerated as colony forming units (cfu) Percentage cell death was
calculated as 100 x [(cfu/ml in cultures without phage) - (cfu/ml in cultures with
phage)] [cfu/ml in cultures without phage]$^{-1}$

Burst sizes were measured as described by Klaenhammer and Sanozsky
(1985) One ml samples were removed at time T0 and at increasing time intervals and
assayed directly for phage using plasmid-free MG1614 as a sensitive host

2.9   Phage DNA replication. Intracellular phage DNA replication was monitored

by the method of Hill *et al* (1991a) DNA samples were digested with *Hin*dIII (ø712) or *Eco*RI (øc2) and electrophoresed on 0 7% agarose gels In the case of phage øc2, the DNA was subsequently transferred to Hybond-N+ nylon membrane (Amersham, UK) by capillary blotting and probed with øc2 DNA using the ECL gene detection system

2.10 Determination of ECOI following electroporation of phage øc2 DNA into hosts. Phage øc2 DNA was electroporated into *L lactis* ssp *lactis* MG1614 and its derivatives and assayed for infective centres by plaquing the electroporated cells on a lawn of the sensitive host MG1614 The efficiency of centre of infection (ECOI) was calculated as [pfu of the electroporated host][pfu of the electroporated sensitive host]$^{-1}$

2 11 Electron microscopic analysis. A phage øc2 preparation was mixed with late-log sensitive and resistant cultures at a multiplicity of infection (moi) of 10 in the presence of 10 mM $CaCl_2$ for 5 min Phage host mixtures were negatively stained with 2% uranyl acetate and examined using a JEOL 1200 EX transmission electron microscope (JEOL, London, UK) at an accelerating voltage of 80 kV

2.12 Rhamnose desorption assay. Five ml of a phage c2 preparation (10^6 pfu/ml) was added to 5 ml of a late-log sample of *L lactis* spp *lactis* MG1614 or its pNP40-containing derivative in the presence of 20 mM $CaCl_2$ A control in which the 5 ml culture was substituted by an equal volume of GM17 broth was also included At 5 min intervals, two 0 5 ml samples of the infected cells were removed and added to either 0 5 ml of quarter strength Ringers solution (Merck, Darmstadt, Germany) or 0 5 ml of 1M L-rhamnose These samples were centrifuged for 4 min and the supernatants assayed for phage Percentage total phage adsorption (reversible and irreversible) was

calculated as 100 x [control titre-residual titre in the Ringers sample][control titre]$^{-1}$ and

% irreversible adsorption was calculated as 100 x [control titre-residual titre in the

rhamnose sample][control titre]$^{-1}$

CHAPTER III

A LATE-ACTING ABORTIVE INFECTION BACTERIOPHAGE RESISTANCE MECHANISM FROM THE LACTOCOCCAL PLASMID pNP40 IS ENCODED BY TWO OVERLAPPING GENES.

3.1   INTRODUCTION

Bacteriophage interference with dairy fermentations can cause slow acid production by the starter culture which can result in an inferior grade product or, in some extreme cases, complete starter failure Numerous strategies have been employed to exclude phage from industrial fermentations and/or to minimise their impact on the cultures employed It is obvious that a number of strains have a natural insensitivity to phage and the DNA responsible for this ability has in many cases been located to plasmids Furthermore, many of these plasmids were found to be conjugative, including pNP40 (McKay and Baldwin, 1984) and pTR2030 (Klaenhammer and Sanosky, 1985) This property has been exploited to develop, using non-recombinant conjugal strategies, improved starter cultures for the dairy industry which are highly resistant to phage (Harrington and Hill, 1991, Sing and Klaenhammer, 1986) However, prolonged use of pTR2030 transconjugants in industrial fermentations has promoted selection of a number of phage which are insensitive to the pTR2030-encoded mechanisms (Hill *et al* 1991b, Alatosava and Klaenhammer, 1991) Rotation of starters containing alternative phage resistance plasmids, or the use of strains containing multiple phage resistance plasmids with complementary mechanisms, can alleviate the pressure on individual plasmids The success of these strategies depends upon the discovery of novel phage resistance genes encoding functionally distinct resistance mechanisms

The conjugative resistance plasmid pNP40, as originally described by McKay and Baldwin (1984), did not conform to the criteria which defined either adsorption inhibition or restriction/modification (R/M), the only phage resistance mechanisms recognised in lactococci at that time Thus, it became the first in a new class of phage resistance plasmids, encoding what is now termed abortive infection (Abi) Several phage resistance plasmids have since been confirmed as encoding Abi mechanisms, e g pTR2030 (Klaenhammer and Sanozsky, 1985), pTN20 (Durmaz *et al* , 1992),

pCI829 (Coffey *et al*, 1989), pIL105 (Gautier and Chopin, 1987) and pCI750 (Murphy *et al*, 1988), many of which have been described as encoding complete resistance to small isometric-headed phage and partial resistance to prolate-headed phage pNP40 is the only lactococcal plasmid, to date, which confers complete insensitivity to both morphological types

This study was undertaken to elucidate the mechanism(s) by which pNP40 mediates this impressive degree of resistance and to analyse at the DNA sequence level the gene(s) encoding insensitivity The cloning and DNA sequence analysis of a 2 5 kb fragment of DNA which confers resistance to the small isometric-headed phage ø712 is described and the stage in the lytic cycle at which this system operates is investigated

3.2   RESULTS

3.2.1 Cloning of a phage resistance mechanism from pNP40

L lactis ssp *lactis* MG1614 containing pNP40 displays complete resistance to øc2 and ø712, as manifested by a complete absence of plaques when an undiluted phage stock (10^9 pfu/ml) is plaqued on a lawn of cells In order to localise the phage resistance determinant(s) within pNP40, a number of *Eco*RI and *Nco*I fragments were cloned into the lactococcal-*E coli* shuttle vector pAM401 Following the introduction of the various recombinant derivatives into *L lactis* ssp *lactis* MG1614, the clones were examined for their ability to confer resistance to phage pPG01, a recombinant plasmid containing a 6 0 kb *Nco*I fragment from pNP40, conferred partial resistance to ø712 (EOP=10^{-4}) No resistance was evident against øc2 A detailed restriction map of pPG01 was generated and subsequent deletion and subcloning analysis permitted the construction of the derivatives pPG03, pPG05, pPG06 and pPG07 (Fig 3 1A and Table 3 1) Examination of the effect on the plaquing efficiency of ø712, when they

46

A
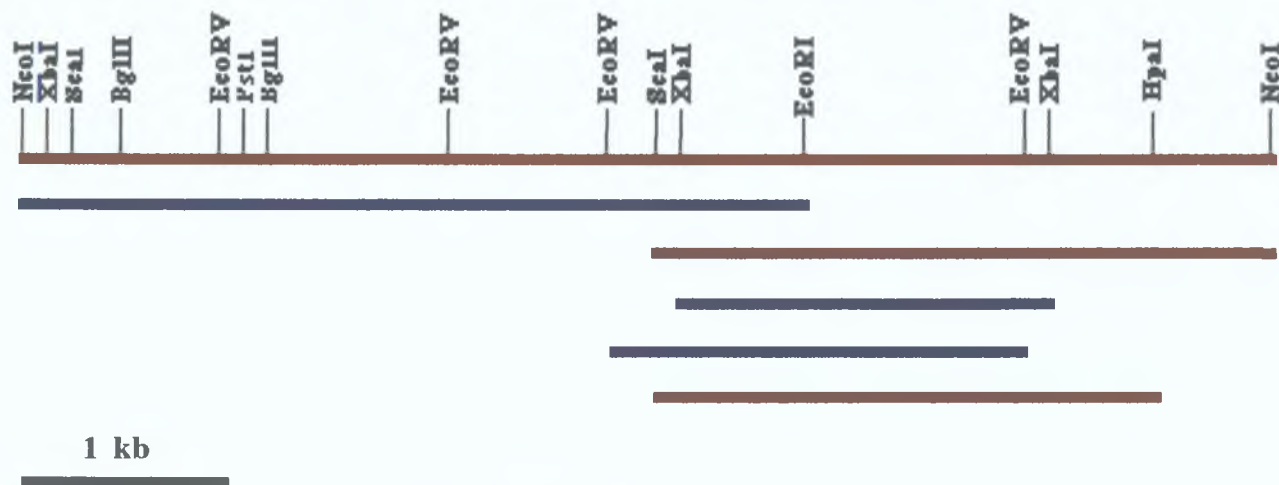
pPG01   ø712   R

pPG03   S

pPG05   R

pPG06   S

pPG07   S

pPG09   R

1 kb

denotes resistant clone; denotes sensitive clone

B

P

abiEi   *abiEii*

(861 bp)   (894 bp)

P denotes putative promoter

Table 3.1

Plasmids

Plasmids	Relevant characteristics	Reference
pAM401	Lactococcal-*E coli* shuttle vector	Wirth *et al* (1986)
pNP40	65 kb plasmid from *L lactis* ssp *lactis* biovar *diacetylactis* DRC3	McKay and Baldwin (1984)
pPG01	6 0 kb *Nco*I fragment from pNP40 cloned into pAM401	This Chapter
pPG03	pPG01 with 2 5 kb *Eco*RI fragment deleted	This Chapter
pPG05	pPG01 with 2 small *Sca*I fragments deleted	This Chapter
pPG06	1 9 kb *Xba*I fragment from pPG01 cloned into pAM401	This Chapter
pPG07	2 1 kb *Eco*RV fragment from pPG01 cloned into pAM401	This Chapter
pPG09	pPG05 with *Nco*I-*Hpa*I fragment deleted	This Chapter

were introduced into *L lactis* ssp *lactis* MG1614, allowed the phage resistance locus to be defined within a 3 1 kb *ScaI-NcoI* region on pPG05 (Fig 3 1A) Further deletion analysis of pPG05, resulting in the plasmid pPG09, identified a 2 5 kb *ScaI/HpaI* fragment as the smallest fragment capable of encoding insensitivity

3.2.2 DNA and deduced amino acid sequence analysis

M13 clones were generated in both orientations encompassing the entire pNP40-derived DNA insert of pPG09 Where overlapping clones were not obtained, pPG01 DNA was used as a sequencing template Sequencing was initiated using commercial M13 primers and continued using synthetic 17-mer oligonucleotides Computer analysis of the sequence data suggested the presence of two overlapping open reading frames (Fig 3 1B)

The 861 bp ORF1 initiates at an ATG start codon at nt 198 and terminates at nt 1058 (Fig 3 2) It has the capacity to encode a protein of 287 aa with a predicted molecular mass of 33 8 kDa Four bp upstream of the ATG start codon, ORF1 is preceded by a putative ribosome binding site (AAAGGAG) with a ΔG value of -14 0 kcal/mole complementary to the lactococcal 16S rRNA The 894 bp ORF2 initiates at an ATG start codon at nt 1058, overlapping the C-terminus of ORF1 by 1 bp, and terminates at nt 1951 It has the capacity to encode a protein of 298 aa in length with a predicted molecular mass of 35 4 kDa A putative ribosome binding site (GGAG), with a ΔG value of -9 4 kcal/mole, precedes ORF2 13 bp upstream of the ATG start codon A consensus -10 sequence (TATAAT) was identified 38 bp upstream of the RBS of ORF1 and was separated by 17 bp from a -35 sequence (TTGTGT) which partially resembles the -35 sequence of the *E coli* consensus promoter (TTGACA) However, no dinucleotide TG was located upstream of the -10 region as has been identified in the promoters of many lactococcal genes (de Vos *et al*, 1987) No consensus -10 or -35 regions were identified upstream of ORF2 suggesting that both ORF's may be transcribed as a polycistronic operon using the promoter sequences preceding ORF1

```
  1                                                    AAGTACTCTTGTTGTATAATGGTAAAAAAATGTATTATT
 40 AATTCAGGAATCATGATTAAATATAATTTATCAAAAAGCTCAATCTGTGAATCATAATTTCTGTCAATTATATAAATAT
119 ATTGTGTTTTTATATATTTTTTTATATAATTGACGTGTATACGCCATGATTCAGGAGTCAAGTGTATAAAAGGAGAAAA
         -35                    -10                                         RBS
```

```
         Met Asp Thr Tyr Arg Ile Ser Asn Leu Asn Leu Glu Gln Glu Asp Ile Asn Asn Leu Lys      20
198 ATG GAC ACT TAT AGA ATA TCA AAC TTA AAT CTA GAA CAG GAA GAT ATA AAT AAT CTA AAG

         Arg Ile Ser Thr Asn Met Leu Asp Thr Phe Asn His Glu Gln Leu Leu Ser Ile Ile Asp      40
258 AGA ATC TCA ACC AAT ATG TTA GAT ACT TTT AAC CAT GAA CAG CTT CTT TCA ATC ATT GAT

         Val Met Lys Asn Thr Tyr Phe Met Asn Glu Leu Ser Thr Tyr Leu Val Asp Asp Asn Leu      60
318 GTG ATG AAA AAT ACT TAT TTC ATG AAT GAA CTC TCA ACC TAT TTA GTA GAC GAT AAT CTC

         Pro Asp Val Gly Thr Glu Glu Phe Asn Phe Leu Val Leu Ala Asn Lys Tyr Lys Gly Asn      80
378 CCT GAT GTA GGT ACT GAA GAA TTT AAT TTT TTG GTT CTT GCT AAT AAA TAT AAA GGA AAT

         Ile Ile Arg Lys Ile Val Arg Asp Glu Gly Ile Ser Asp Tyr Tyr Leu Arg Lys Phe Val     100
438 ATA ATA AGA AAA ATA GTA CGT GAT GAA GGT ATA AGT GAT TAC TAT TTG AGA AAA TTT GTT

         Leu Lys Tyr Asn Leu Thr Glu Val Asp Lys Gly Val Tyr Ile Phe Pro His Lys Lys Lys     120
498 TTG AAG TAT AAT CTT ACT GAG GTT GAC AAA GGT GTT TAT ATT TTT CCT CAT AAA AAA AAG

         Asp Ser Leu Phe Ile Phe Gln Gln Lys Tyr Ser Lys Ala Val Ile Ser His Glu Thr Ser     140
558 GAC AGT TTA TTT ATT TTT CAG CAG AAA TAC AGC AAA GCA GTT ATC TCA CAT GAA ACA TCA

         Leu Tyr Leu Gln Asp Val Ile Asp Tyr Ile Pro Gln Lys Ile Gln Met Ser Val Pro Glu     160
618 TTA TAT CTA CAA GAT GTA ATA GAT TAT ATT CCG CAA AAA ATA CAA ATG AGC GTT CCA GAA

         Lys Tyr Asn Ile Ser Arg Ile Gln Glu Pro His Glu Asn Arg Leu Thr Ser Tyr Asn Tyr     180
678 AAG TAT AAT ATC AGT AGA ATT CAG GAA CCT CAC GAA AAT CGT TTA ACA AGC TAT AAC TAT

         Val Asp Ile Asn Ser Asn Asn Ile Met Asp Lys Asn Ile Pro Ile Asn Leu Val Arg Asn     200
738 GTG GAT ATT AAC TCT AAT AAT ATA ATG GAT AAA AAT ATT CCA ATC AAT TTA GTC AGA AAT

         Lys Ser Ile Ser Pro Thr Gln Ile Glu Thr Val Asn Ser Phe Leu Gly Leu Pro Leu Arg     220
798 AAG AGT ATT AGT CCT ACA CAA ATA GAA ACA GTA AAT AGC TTT TTA GGT CTC CCA CTA AGA

         Val Thr Ser Ile Ala Arg Ser Ile Val Asp Val Leu Lys Pro Ser His Lys Ala Glu Glu     240
858 GTC ACT TCT ATT GCT CGG TCG ATA GTA GAC GTT TTA AAA CCT TCC CAC AAG GCT GAA GAA

         Glu Val Lys Glu Gln Ala Ile Lys Tyr Tyr Leu Glu Arg Phe Pro Asp Asn Ile Val Arg     260
918 GAA GTG AAA GAA CAG GCG ATT AAG TAT TAT TTA GAA AGA TTT CCA GAT AAT ATT GTG CGC

         Leu Lys Arg Ile Ala Lys Thr Gln Asn Val Leu Lys Glu Leu Glu Tyr Tyr Leu Ile Leu     280
978 TTA AAA CGT ATA GCT AAA ACA CAA AAT GTT TTA AAA GAA CTA GAG TAT TAC TTG ATT TTA

         Leu Gly Val His Tyr Lys Leu Stop                                                     287
                                            Met  Lys Asn Thr Arg Leu Lys Asp Leu Ile Ala Thr Arg     13
1038 TTG GGA GTA CAT TAT AAA CTA TG  AAA AAT ACA AGG TTA AAG GAT TTA ATT GCA ACA AGA
         RBS

         Asn Asp Asp Ile Gly Ile Glu Asn Tyr Arg Ile Arg Tyr Ala Thr Glu Arg Phe Leu Thr     33
1097 AAT GAT GAT ATT GGA ATT GAA AAT TAT AGA ATA AGA TAT GCA ACT GAA AGA TTC TTA ACA

         Arg Leu Ser Ala Ser Gln Tyr Lys Glu Lys Phe Val Leu Lys Gly Gly Phe Leu Ile Gly     53
1157 AGG CTT TCG GCA AGT CAA TAT AAG GAA AAA TTT GTT TTA AAA GGA GGT TTT TTA ATT GGT

         Val Thr Tyr Asn Leu Ser Gln Arg Thr Thr Lys Asp Leu Asp Thr Ala Leu Ile Asp Phe     73
1217 GTC ACC TAT AAC TTG AGT CAG AGG ACT ACT AAG GAT TTA GAT ACA GCT CTG ATA GAC TTT

         Lys Ser Asp Ala Gln Ser Ile Glu Arg Val Ile Thr Glu Ile Cys Asn Ile Asp Leu Glu     93
1277 AAA AGT GAT GCA CAG TCT ATT GAA CGG GTA ATT ACT GAA ATA TGT AAT ATA GAT TTA GAA

         Asp Gln Val Leu Phe Lys Leu Lys Glu Leu Thr Ser Ser Gln Asp Met Arg Ile Tyr Pro    113
1337 GAC CAA GTG CTT TTT AAA TTA AAG GAA CTT ACA AGC AGT CAA GAT ATG AGA ATA TAT CCT

         Gly Tyr Arg Ala Lys Leu Lys Met Met Phe Pro Asp Gly Asn Thr Arg Ile Asp Phe Asp    133
1397 GGG TAC AGA GCT AAA CTT AAG ATG ATG TTT CCT GAT GGA AAT ACA AGG ATA GAC TTT GAT

         Leu Asp Ile Gly Val Gly Asp Arg Ile Thr Pro Glu Ala Lys Lys Ile Lys Ile Pro Leu    153
1457 CTT GAT ATT GGA GTA GGA GAT AGG ATA ACC CCA GAG GCT AAG AAA ATA AAA ATA CCT CTA

         Ile Phe Asn Glu Val Lys Gly Val Glu Lys Gln Ile Glu Val Leu Ala Tyr Pro Lys Glu    173
1517 ATA TTT AAT GAA GTA AAA GGT GTA GAG AAA CAG ATA GAA GTA TTA GCT TAT CCA AAA GAA

         Thr Ile Gln Ala Glu Lys Leu Glu Thr Ile Leu Thr Arg Gly Lys Val Asn Thr Arg Met    193
1577 ACT ATT CAG GCA GAA AAA TTA GAG ACA ATT CTC ACT CGG GGG AAA GTA AAT ACT AGA ATG

         Lys Asp Tyr Tyr Asp Phe His Leu Leu Leu Thr Asp Gln Glu Asn Ser Asn Ser Ile Ser    213
1637 AAA GAT TAT TAC GAT TTT CAT TTG CTT TTG ACT GAT CAA GAA AAT AGT AAT AGT ATA AGT

         Phe Tyr Tyr Ala Phe Lys Asn Thr Trp Glu Phe Arg Asn Pro Thr Gln Phe Ile Asp Glu    233
1697 TTT TAT TAT GCT TTT AAA AAT ACT TGG GAA TTT AGA AAC CCA ACT CAA TTT ATT GAT GAA

         Glu Leu Phe Glu Asp Trp Leu Phe Ile Leu Asp Glu Ile Leu Glu Ser Lys Glu Leu Lys    253
1757 GAA CTG TTT GAA GAT TGG CTC TTT ATT TTA GAT GAG ATA CTT GAA TCA AAA GAA TTG AAA

         Glu Lys Tyr Trp Pro Asn Tyr Ile Lys Asp Arg Asn Tyr Ala Lys His Leu Asn Met Asp    273
1817 GAA AAG TAT TGG CCA AAC TAT ATT AAA GAT AGA AAT TAT GCT AAA CAT CTA AAC ATG GAT

         Asp Ile Ile Ser Glu Ile Lys Glu Phe Val Ser Lys Leu Lys Glu Glu Tyr Ile Lys Glu    293
1877 GAT ATC ATT TCA GAA ATT AAA GAA TTT GTT AGT AAA TTA AAA GAA GAA TAT ATA AAG GAG

         Asn Met Ser Arg Asn Stop                                                            298
1937 AAT ATG TCT AGA AAT TAA TAAAGATTATAAGAACAATATTTTTAAGTTGTTATTTTTATATCATAATTGACGA
```

```
2010 ACTTGTATCCTTCAATTCCTATAAAATCAACCAAGTTTCAAGAATACTATTCTTTTTCGTTAAGTTAATTGAGATGGGAA
2089 AAAAGGGGGCTAAATTTAGTCCCCTTTTATTTATGAAAATTGCAAAGCTTCACGAGTTAAGTTATCTAGTGCATTAAAAA
```

ΔG=-25.2 kcal/mole

```
2168 CCGAAGGATGATGAGAGAGATATTCTTTTTACTCCGAAAATTCCACGTCCTAATTTTTTGTCCATTAAATGAATACCATTC
2247 GCCTAGCTTCTTTACTACTCCAAGTTGAAGTGCAGTTGTAACGACATCAATTTCTTGAGAAAATCCGTCTCCAAAAATA
2326 TTAATAACAGTTGGCTTTTTATAGGGCGATGAAACCTTATTTTTTGACTGTATGAAGTGTTGTTTTTTTTACCAATGGTAT
2405 CTATTCCATCTTTAATTTTTTCTCCAGATTTCACTTCGATACGAACAGATGAATAGAATTTTTAGAGCCGAACCACCTGG
2484 GGTGGTTTCTTTATTTAAGAACAAGCCACTCATGGTAGAGCGTATTTGGTTGATGAAGATGATTACTGTCTTTGTTTGG
2563 TTAAC
```

The nearest structure which could function as a transcriptional terminator is a 14 bp inverted repeat sequence (ΔG=-25 2 kcal/mole) which is located 135 bp downstream

The smallest clone which confers insensitivity to phage contains both ORF1 and ORF2 (i e pPG09) pPG07, which contains all of ORF1 but is missing 69 bp at the C-terminus of ORF2, does not confer resistance to ø712 pPG06, which encodes all but the C-terminal aa of ORF2 but lacks the promoter sequences preceding ORF1 and the N-terminal 10 aa of ORF1, is also sensitive to ø712 This infers that either both ORF's are required for expression of the resistance phenotype or that ORF2 alone is sufficient but requires DNA sequences upstream of ORF1 for expression

The GC contents for both ORF1 and ORF2 were 29% which is low compared to the 37% average for lactococcal genes, but is characteristic of lactococcal *abi* genes in general Comparative analysis with known sequences in the EMBL and Genbank databases (release 82), using the DNAstar software program, revealed no DNA sequence homology

Similarly, comparative analysis of the deduced amino acid sequence with known sequences in the Swiss and PIR and Translated databases (release 82), revealed no significant amino acid sequence homology A search of both deduced amino acid sequences for functional motifs from the Prosite database (release 11), using the MacPattern and DNAstar (DNAstar INC Madison, WI) programs, gave no indication of homology, offering no indication of a possible basis for this mechanism

3 2.3 Insensitivity encoded by pPG01

A number of assays were performed to assess the nature of the resistance encoded by pPG01 and by pNP40 Plaque assay results indicated that the EOP of ø712 on *L lactis* ssp *lactis* PG001 was 10-4 relative to plasmid-free *L lactis* ssp *lactis* MG1614 (Table 3 2) No accompanying reduction in plaque size was observed As this phenotype could reflect the operation of an R/M system, host-dependent plaquing efficiency was assayed Phage from plaques obtained on PG001 were found to plaque

Table 3.2

Plaquing efficiency of ⌀712 on *L. lactis* ssp. *lactis* MG1614 and PG001

Phage	MG1614	PG001
MG1614	1 0	4 0x10^{-4}
MG1614 PG001	1 0	7 3x10^{-1}
MG1614 PG001 MG1614	1 0	1 2

more efficiently on PG001 in a second round of plaquing (Table 3 2) This increased efficiency was not reversible by passage through MG1614 indicating that the ability of these phage to plaque on PG001 is host-independent Therefore, pPG01 presumably does not encode an R/M system and these phage represent mutants which are not affected by the pPG01-encoded mechanism As previously stated, no plaques were obtained on cells containing pNP40 To elucidate the type of resistance encoded by pPG01, a number of further assays were performed

The presence of pPG01 did not affect the ability of *L lactis* ssp *lactis* MG1614 to adsorb ø712 (Table 3 3) Over 97% of the phage adsorbed to plasmid-free MG1614 and to PG001, demonstrating that the phage resistance mechanism acts post-adsorption

Examination of the number of cells which die as a result of infection is a valuable indicator of whether the resistance mechanism acts early, e g adsorption inhibition or R/M, or late in the lytic cycle, i e abortive infection Cell death results indicated that 85% of pPG01-containing cells died as a result of ø712 infection When pNP40 was present in the host, cell death remained relatively high at 42% (Table 3 3) ECOI assays, which indicate the number of infected cells which give rise to productive infections, were carried out on sensitive and resistant hosts By convention, it is assumed that 100% of infected sensitive cells result in progeny phage (ECOI=1 0) Only 15% of cells containing pPG01 resulted in productive infections This was reduced to 3% in cells containing pNP40 (Table 3 3) The burst size for ø712 propagated on PG001 was considerably reduced with only 7 viable progeny per cell as compared to 54 per cell following infection of the plasmid-free host Burst size could not be evaluated in cells containing pNP40 due to the low ECOI (Table 3 3) The high cell death result, which is characteristic of Abi, indicated that pPG01 mediates resistance to ø712 by an Abi-type mechanism In addition, reduced ECOI and burst size results are typical of abortive infection phenotypes The mechanism encoded by pPG01 was designated AbiE in keeping with the nomenclature suggested by Coffey *et al*

53

Table 3.3

% Adsorption, and % cell death, ECOI and burst size of ⌀712 on *L. lactis* ssp. *lactis* MG1614, PG001 and MG1614/pNP40

Strain	Resistance	% adsorption	% cell death	ECOI	burst size
MG1614	none	97 0	100	1 0	54
PG001	AbiE	97 1	85	0 15	7
MG1614/pNP40	total	97 3	42	0 03	np

ECOI-Efficiency of centre of infection
np- not possible to evaluate

(1991) and ORF1 and ORF2 were consequently designated *abiEi* and *abiEii*

3.2.4 Effect of AbiE on ø712 DNA replication

The replication of phage DNA in sensitive and resistant hosts was examined at 15 minute intervals following ø712 infection (Plate 3 1) Normal phage development was monitored in plasmid-free hosts with the infecting phage genomes detected within the cell after 15 minutes The intracellular phage DNA concentration increased over time until 60 minutes when a considerable reduction was observed This presumably reflected phage DNA packaging and release of progeny In the cells expressing AbiE, the DNA of the infecting particles entered the cells as normal during the first 15 minutes and there was little effect on the level of intracellular phage DNA replication However, there was no evidence of DNA packaging or phage release as was discerned in the sensitive host after 60 minutes, suggesting that AbiE operates late in the lytic cycle This corroborates the previous results which suggested that AbiE operates by an abortive infection-type mechanism In MG1614/pNP40, phage DNA replication was completely inhibited with no increase in phage DNA concentration following internalisation (Plate 3 1)

3.3   DISCUSSION

Bacteriophage resistance mediated by abortive infection is widely distributed in *Lactococcus* This paper describes the cloning of an abortive infection mechanism (AbiE) from pNP40 which confers resistance to ø712 by interfering in phage development late in the lytic cycle This system is unusual in that two ORF's are apparently required for expression of the resistance phenotype In addition, the parental plasmid pNP40 confers resistance to øc2 indicating the operation of at least another phage resistance mechanism other than AbiE.

Plate 3.1. DNA content of *L lactis* **ssp.** *lactis* **MG1614, PG001 and MG1614/pNP40 hosts following infection with ø712.** Lanes 1 to 6 show the *Hind*III-digested total DNA isolated at 0, 15, 30, 45, 60 and 75 minutes, respectively, from ø712-infected MG1614, PG001 and MG1614/pNP40 (panels A, B and C, respectively)

A
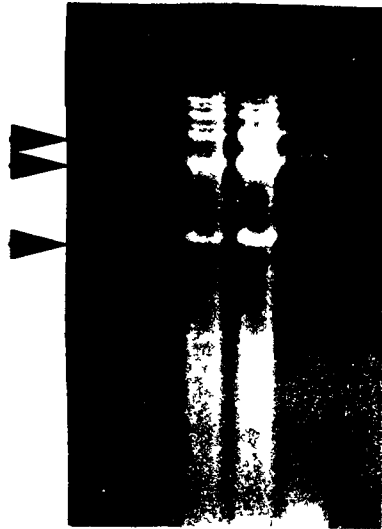
1 2 3 4 5 6

B

1 2 3 4
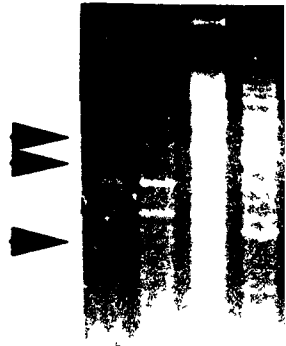
C

5 6   1 2 3 4 5 6
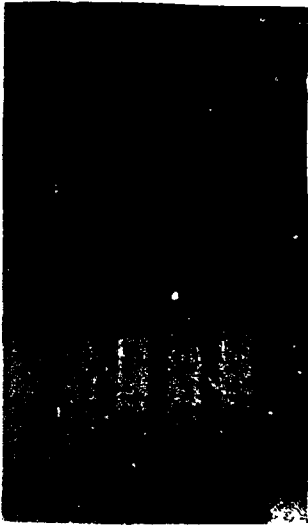
The phage resistance phenotype mediated by pPG01 conforms to the definition of abortive infection Phage adsorbed to the host normally, their DNA was injected and the host cells were killed Phage DNA replication occurred but no evidence of packaging of phage DNA or phage release were observed In fact, 85% of infected cells did not produce progeny phage and the burst size in the remainder was reduced 8-fold The majority of phage did not form plaques on hosts expressing AbiE, however, mutant phage were detected at a frequency of 10^{-4} and formed normal-sized plaques These phage plaqued with equal efficiency on MG1614 and PG001 regardless of the host used for propagation

The parental plasmid pNP40 confers complete resistance to $\phi712$ with no plaques detected even when phage titres of $>10^9$ pfu were employed Phage $\phi712$ adsorbed to MG1614/pNP40 and phage DNA entry into the cell was normal The presence of pNP40, however, completely inhibited phage DNA replication, reducing the ECOI and burst size compared with that obtained for AbiE alone This enhanced level of resistance reflects the operation of a second phage resistance mechanism which, when combined with AbiE on pNP40, is responsible for this phenotype (Chapter 4)

When first coined, abortive infection was used to specify phage resistance mechanisms which reduced the efficiency of plaquing and/or plaque size It is now accepted that, despite superficially similar phenotypes, Abi describes a variety of late-acting phage resistance mechanisms Investigations have shown that AbiA (pTR2030) functions by preventing phage DNA replication (Hill *et al*, 1991a) which results in negligible production of structural gene products such as the major capsid protein (MCP) (Moineau *et al*, 1992) Conversely, AbiC(pTN20) has no effect on ϕDNA replication but interferes in transcription/translation processes, decreasing production of the MCP by 50% (Moineau *et al*, 1992)

The observations for AbiE suggest a late target of inhibition as there is little effect on phage DNA replication Possible mechanisms include interference in transcription/translation processes, as in the case of AbiC (Moineau *et al*, 1992), or

defects in packaging or release of phage particles In gram negative bacteria,
deficiencies in late protein synthesis have been observed with a number of Abi systems
The Pif system encoded by the F factor in *E coli* induces ribosomal misreading which
results in suppression of ochre stop codons (Kruger and Bickle, 1987) This gives rise
to inaccurate translation of late phage genes and aborts the infection In *Vibrio cholerae*
biotype El Tor hosts, deficiencies in transcription/translation were shown to be due to
destabilisation of phage DNA concatemeric replication intermediates Association of
phage DNA with the cell membrane is a requirement for late protein synthesis in
permissive *Vibrio* hosts This is hindered in El Tor cells by a small hydrophobic protein
which inserts itself into the cell membrane (Chowdury *et al* , 1989) The effect, if any,
of AbiE on phage protein synthesis remains to be established

The genetic organisation of AbiE is striking in that it appears that two
overlapping ORF's may encode the abortive infection phenotype Overlapping genes
are not unusual in lactococci where they have been found extensively among ORF's in
amino acid biosynthesis operons, among others (Chopin, 1994) However, it is a
unique arrangement among the lactococcal abortive infection genes sequenced to date,
all of which involve a single ORF Overlapping of genes may induce translational
coupling which is believed to direct the stoichiometric synthesis of proteins by making
translation of the second gene dependent on that of the first, thus ensuring proportional
production of proteins The absence of an identifiable promoter for *abiEii* substantiates
the possibility of co-transcription for these ORFs Analysis of the mRNA content of
resistant hosts could confirm this Interestingly, the *E coli* Pif system is also encoded
by two genes, *pifA* and *pifB*, which may be transcribed from a single promoter (Cram
et al , 1984)

Another striking feature of this system is the low GC contents of *abiEi* and
abiEii This is also true of *abiA*, *abiB* and *abiC*, all of which have GC contents of 27%
It is not known if these atypical GC contents reflect the function performed by *abi*
genes or if it reflects their origin No homology was detected at the DNA or amino acid

sequence level between any of the abortive infection genes which have been analysed to this degree, except *abiA* from pTR2030 and pCI829 which were found to be identical (Coffey *et al*, 1991) Although the molecular basis of Abi phage interactions has not been established, the results to date reflect the diversity and potential of abortive infection mechanisms Thus, AbiE represents a further example of phage resistance which may be used alone or in combination with other phage resistance mechanisms in the construction of improved starter cultures

CHAPTER IV

CLONING AND DNA SEQUENCE ANALYSIS OF AbiF-A SECOND ABORTIVE INFECTION MECHANISM FROM THE LACTOCOCCAL PLASMID pNP40

4.1 INTRODUCTION

Several lactococcal phage resistance plasmids have been identified to date which encode two phage resistance determinants The most frequently observed combination is restriction/modification (R/M) and abortive infection, e g pTN20 (Durmaz *et al*, 1992), pTR2030 (Hill *et al*, 1989a) and pBF61 (Murphy *et al*, 1988) Adsorption inhibition has also been found in association with Abi, e g pCI528 (Costello, 1988), and with R/M, e g pAH90 (Harrington and Hill, 1992) The advantages conferred on a host by combining phage resistance mechanisms are twofold, either the level of resistance mediated against a particular phage and/or the range of phage to which it is insensitive may be increased

Based on the results presented in Chapter 3, it appears that pNP40 encodes at least one more system in addition to AbiE, enabling it to confer resistance to øc2 in addition to ø712 This study was undertaken to elucidate the mechanism(s) by which pNP40 confers resistance to øc2 It describes the cloning and DNA sequence analysis of a second phage resistance gene from pNP40, and investigates the stage in the lytic cycle at which it mediates resistance to phage Furthermore, it provides evidence for the existence of a third phage resistance mechanism on pNP40 also operational against øc2

4.2 RESULTS

4.2.1 Cloning and localisation of a phage resistance gene from pNP40

In Chapter 3, we described a locus on pNP40 which mediates resistance to ø712 by an abortive infection mechanism, designated AbiE The ability of pNP40 to mediate resistance to øc2, in addition to ø712, suggested the presence of a second phage resistance determinant on this replicon In order to localise this determinant, a number of *Eco*RV fragments of pNP40 were cloned into the lactococcal-*E coli* shuttle

vector pAM401 Clones were initially selected in *E coli* HB101 before being introduced into *L lactis* ssp *lactis* MG1614 where they were screened for their ability to confer resistance to phage A single recombinant plasmid, pCG1, harbouring a 5 6 kb insert, conferred partial resistance to both øc2 and ø712 A detailed map of pCG1 was generated and subclonmg and deletion analysis of pCG1 enabled the construction of several derivatives, pPG21-pPG25 (Table 4 1 and Fig 4 1A), which were subsequently introduced into *L lactis* ssp *lactis* MG1614 Assessment of their effect on the efficiencies of plaquing (EOP's) of øc2 and ø712 allowed the phage resistance gene to be localised to a 3 7 kb *Xba*I-*Eco*RV fragment on pPG23 (Fig 4 1A) In addition, the introduction of an additional 4 bp at the unique *Bgl*II site on pCG1, by the activity of the Klenow fragment of DNA polymerase I on *Bgl*II digested pCG1 DNA, generating the derivative pPG26, disrupted the phage resistance phenotype

4.2.2 DNA and deduced amino acid sequence analysis

M13 clones were generated in both orientations which encompassed most of the pNP40-derived DNA insert of pPG23 Where overlapping clones were not obtained, pCG1 was used as a sequencing template Computer analysis of the sequence data suggested the presence of a single complete ORF which crosses the *Bgl*II site (Figs 4 1B and 4 2) This ORF initiates at nt 1405 and terminates at nt 2430 and is therefore 1026 bp in length It has the capacity to encode a protein of 342 aa with a predicted molecular mass of 41 2 kDa Six bp upstream of the ATG start codon, the ORF is preceded by a putative RBS (AAAGG) with a ΔG value of -9 6 kcal/mole A -10 sequence (TTTAAT) was identified 85 bp upstream of the RBS and was separated by 17 bps from a -35 sequence (TTGAAT) partially resembling the -10 and -35 sequences (TATAAT and TTGACA, respectively) of the *E coli* promoter A TG dinucleotide found in many lactococcal promoters is located 1 bp upstream from the -10 sequence An 8 bp inverted repeat, with a ΔG value of -13 4 kcal/mole, 2 bp downstream of the ORF has the potential to form a stem-loop structure and thus, could serve as a

Table 4.1

Plasmids

Plasmids	Relevant characteristics	References
pAM401	Lactococcal-*E coli* shuttle vector	Wirth *et al* (1986)
pNP40	65 kb plasmid from *L lactis* ssp *lactis* biovar *diacetylactis* DRC3	McKay and Baldwin (1984)
pPG01	6 0 kb *Nco*I fragment from pNP40 cloned into pAM401	Chapter 3, this thesis
pCG1	5 6 kb *Eco*RV fragment from pNP40 cloned into pAM401	This Chapter
pPG21	pCG1 with *Bam*HI-*Bgl*II fragment deleted	This Chapter
pPG22	pCG1 with 2 *Pst*I fragments deleted	This Chapter
pPG23	3 7 kb *Eco*RV-*Xba*I fragment from pCG1 cloned into pAM401	This Chapter
pPG24	1 8 kb *Xba*I fragment from pCG1 cloned into pAM401	This Chapter
pPG25	4 0 *Bam*HI-*Bgl*II fragment from pCG1 cloned into pAM401	This Chapter
pPG26	pCG1 containing frame shift mutation at *Bgl*II site	This Chapter

A

	ØC2	Ø712
pCG1	R	R
pPG21	S	S
pPG22	S	S
pPG23	R	R
pPG24	S	S
pPG25	S	S
pPG26	S	S

1 kb

■ denotes resistant clone; ■ denotes sensitive clone

B

abiF

(1026 bp)
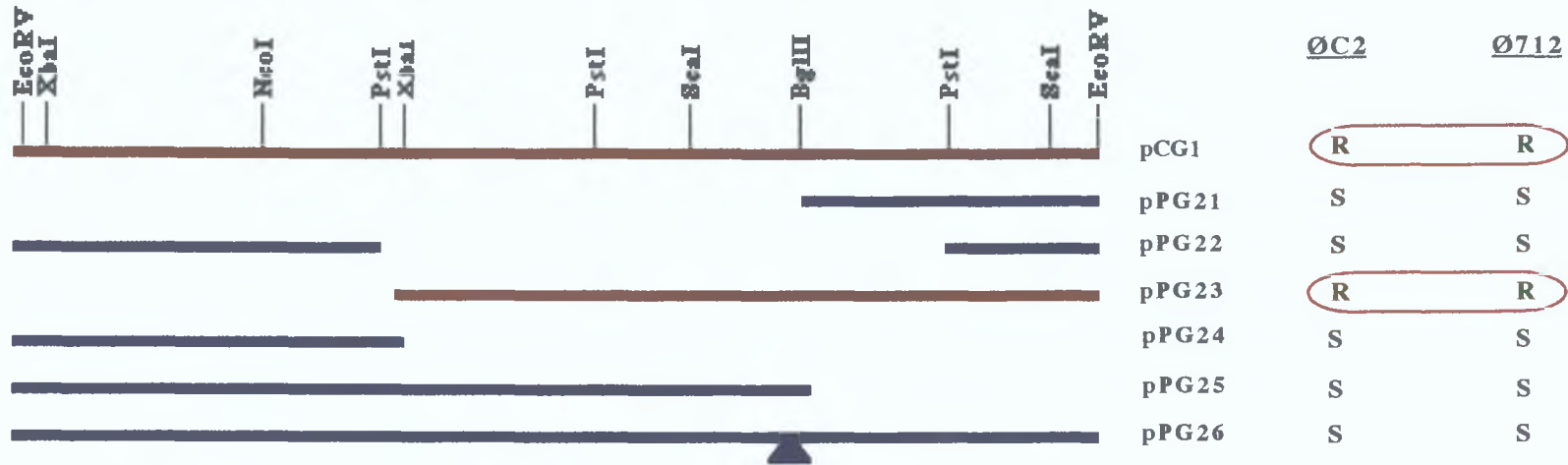
P
└→ denotes putative promoter and T denotes putative terminator

■ denotes region of secondery structure

```
   1                    TCTAGAAACTGAGAGTGACTTTTTATAATCAGCCATTGAAAATCCCACAAAGAGCGAAAAA
  62 TTTGTCGCTTTTTTTCTTTGATTTTCTTAGTCTCACAGCAACTTGTTCAGCCATTTCATTTATTTATTCATCCTATTTCCG
 141 ATTTACGTGTATAATCTCTAGGTAGGGTTTGACTATTTGAGAAACTGACAGATTTCCTTTTATACTTGTCAGTTAAACG
 220 TGTTTCATCAATTCCGTTAGCATGAAAAAATTGTTGAAGCCCAATCACTCCTAATTCACGCTTTAACATATCAGGGTCA
 299 GCATGAGCAAGCTCTTTTATTGAATGAATCCCCAACTTATTTAAACGTGCTTCAGTTCTTACATTAATACCCCAAAAGT
 378 CAGTCATATCAGAGATTGACCACACCTTAGAGGGAACATCTTCATAGCGAATCAAGGCTCTCATGTTGGTATTATGTTT
 457 AGCATAATTATCCATTGCAAGTTTTGCGAGAAGTGGATTGTCTCCCATTCCAATTGTCACATATAAGCCAGTTTTATGA
 536 TAAATTTTACGCTGCAGCATTTGAGCTAACTTATCCATTTGTTCGTATGTATTAGTAATTTCAGGAAAGAAGAAGTCCA
 615 AAGATTCGGTAACATCTAAACAGGATTCATCTATAGAGTAAGCATGGATTTCATCTATAGAGGTAATTTCTCTCAAAAT
 694 ATTGATTACTTCTAAATTTTTTTTGATATATAGCAGCATTTGAGGAGGAACAATATAAGTTTGTCTTGCCCAACGTTCA
 773 ACTTCAGAAATATATTTTGGATCAGGTTCTACAGTCTGTCCAAAAATATCTGTATGTTTTTTGTACCATAGCCGATAGT
 852 TAAATTTACGGTTATGTACCAGGAACGGAAGTTCTTTGGAATGACTAACATTAGACATTCCAAATACTTTCTTGAAAGT
 931 TGGACTAGCAGCAAGTGTTAAGCCATTTGAATTATCAGCTCTACTCATTACACAAAGAGAAGTAGTCAGGGGATTTAAC
1010 CCACGTTCAATACATTCAATTGAAGCGTAATTAGATTTAACATCTTCAAAAAAGATAGCTCGACGAGGTTCAAGTGAAT
1089 AGTCAAATTGGTTATTTAGTATTTTGTATTCCCATTTGCACCTCCAGTAGTATATCAAACACAAAATATCTGTTTAAATG
1168 ATATTATACATTGAAAAAGTCCTAAAATCAATATATAATATACGAATAAATAGTAAAACGTTTGGAATTTAAACTTTTC
1247 CAGTAAAAAAAAAACTATTTTTAATTAAAATCTCTTGAATTTCAAACTAAAATATGTTTTAATATATATATCAGTACTTC
                                            35                10
1326 CCACGCCTCTGTTTTTAAAACAAGCGCCATCATGGAAAGACTTTTTTTTTACAATAAAATAGTGTAGTAAAGGTTACTT
                                                                            RBS

     Met Asn Arg Asn Gly Lys Arg Arg Phe Tyr Asn Ser Leu Gly Thr Ser Ile Lys Ile Arg     20
1405 ATG AAC CGA AAT GGA AAG AGA AGA TTT TAT AAT TCA TTA GGT ACT TCA ATC AAG ATA CGT

     Lys Arg Ser Ile Lys Lys Leu Val Ile Glu Asn Gly Leu Phe Lys Glu Gln Arg Val Lys     40
1466 AAA AGA AGT ATA AAA AAA TTG GTT ATC GAA AAT GGA TTA TTT AAA GAA CAG AGA GTA AAA

     Pro Tyr Phe Asp Glu Asp Glu Ile Ile Lys Lys Ile Asn Lys Ser Ile Phe Ile Asn Asp     60
1526 CCA TAT TTT GAT GAG GAT GAA ATA ATA AAA AAA ATC AAT AAA TCA ATT TTT ATT AAT GAT

     Ser Asp Asn Phe Ile Leu Lys Asn Phe Leu His Lys Asn Ser Tyr Phe Arg Phe Asn Ile     80
1586 TCT GAT AAT TTT ATT CTA AAA AAT TTT TTA CAT AAA AAT AGT TAT TTT AGG TTT AAT ATT

     Tyr Val Lys Leu Met Lys Asp Asn Glu Gly Ile Thr Ile Ser Asp Val Ile Arg Thr Tyr    100
1646 TAT GTC AAA TTA ATG AAA GAT AAT GAA GGA ATT ACA ATT TCA GAT GTA ATT AGA ACC TAT

     Gln Leu Asp Glu Phe Ile Arg Glu Asn Leu Phe Ile Phe Ser Thr Arg Leu Glu Ile Phe    120
1706 CAG TTA GAT GAA TTT ATT AGA GAA AAT CTT TTC ATT TTT TCA ACT CGC TTA GAA ATT TTT

     Trp Lys Lys Lys Ile Ile Asp Thr Leu Cys Ala Glu Tyr Gln Glu Ser His Leu Tyr His    140
1766 TGG AAG AAA AAA ATA ATT GAC ACT TTA TGT GCA GAA TAC CAA GAG TCT CAT CTA TAT CAT

     Val Ser Gln Cys Tyr Leu Asp Lys Asp Leu Tyr Ser Gly Asp Glu Trp Gly Gln Lys Val    160
1826 GTA AGT CAA TGT TAT TTA GAT AAA GAT CTA TAT AGT GGT GAT GAG TGG GGA CAG AAA GTA
                                      BglII
     Ile Asn Asp Phe Ser Ser Phe Phe Tyr Thr Asn Lys Ser Pro Asn Phe Lys His His His    180
1886 ATT AAT GAT TTT AGT TCT TTT TTT TAT ACA AAT AAA AGT CCT AAT TTT AAG CAT CAT CAC

     Asn Asp Lys Lys Asn Tyr Leu Pro Ile Trp Ala Leu Val Glu Glu Leu Thr Phe Gly Gln    200
1946 AAT GAT AAA AAG AAC TAT CTA CCA ATT TGG GCT CTA GTA GAA GAG TTA ACT TTT GGT CAA

     Leu Thr Thr Phe Ile Ser Gln Ile Lys Pro Thr Tyr Ser Ser Ala Trp Ala Met Ala Cys    220
2006 CTG ACA ACG TTT ATA AGC CAA ATC AAA CCT ACA TAT TCG AGT GCT TGG GCA ATG GCT TGT

     Tyr Asn Asn Pro Lys Tyr Lys Ser Thr Leu Asn Ser Trp Met Asn Val Val Arg Leu Tyr    240
2066 TAT AAT AAT CCA AAA TAT AAG TCA ACG TTG AAT AGT TGG ATG AAC GTA GTT AGG TTA TAC

     Arg Asn Lys Ser Ala His Gly Ser Arg Ile Phe Gly Leu Lys Ala Val Asn Val Pro Gln    260
2126 AGG AAT AAA AGT GCG CAT GGA TCA AGA ATA TTT GGG TTA AAG GCT GTA AAT GTA CCC CAA

     Ile Ile Arg Lys Asp Phe Lys Tyr Tyr Phe Pro Asn Lys Gln Glu Ala Asn Leu Arg Lys    280
2186 ATA ATA AGA AAA GAT TTT AAA TAT TAT TTT CCA AAT AAA CAA GAA GCT AAC TTG CGA AAA

     Ser Tyr Leu Tyr Gly Ala Leu Tyr Val Phe Lys His Leu Leu Ile Tyr Glu Asp Asn Phe    300
2246 TCC TAT TTA TAT GGT GCA CTA TAT GTG TTT AAA CAT TTA TTG ATT TAT GAA GAT AAT TTT

     Thr Gln Arg Ser Trp Asn Arg Phe Leu Leu Glu Leu Asn Asn Arg Ile Asn Leu Ile Ser    320
2306 ACT CAA AGA AGT TGG AAT AGA TTT TTA CTA GAA TTA AAT AAT CGA ATT AAT TTA ATT TCT

     Gly Leu Asp Gln Asn Leu Tyr Gly Leu Pro Glu Asp Trp Phe Gln Lys Leu Arg Ile Met    340
2366 GGA TTA GAT CAA AAT CTT TAT GGA TTA CCA GAG GAT TGG TTT CAA AAA TTA CGG ATC ATG

     Ile Ile Stop                                                                        342
2426 ATA ATA TAA AAAAAAAAGGATTAAAACGTTTTTTTATATTACTCTTAAAGATAAATATATTTAACTTTTATTTTTTC

              AG=-13 4 kcal/mole
2502 ATTTTGTATTATAGAATTAAAAATCAGCCTTAAGTCTTAGAAATACATGTAAGTGATGTGTTATAATTAATGTTGCTAG

2581 GATGAATATTCCTATAAGATTACTTTTAAACGAAACAAACTAAGAAATCAACACAAACTAAACTAATAAACTAACACAA

                                DR1                                      DR2
2660 ACTAAACTAATAAACTAACACAAACTAAACTAAGAAATCAAAGAAGAATGACAATTATGAAATATATAGAGTTTAGTAA

              DR3                 DR4
2739 TCTCGTCGGGTAAAACATCCTAAATTTATAGTTTAGGAGTATCGTAATGAAAGTTAGTTTACAAATTAATTCATTCAAA

              AG= -9 4 kcal/mole
2818 TTCAATTTTGAAATAGATTGTAAAGTTATTGCTATGTTACTAGCATTCTTCAAGTAATTAAATAGAATAGGAGGCTTTT
2897 TGCCTTCTATTTTTTTCTTTAATAAAATTTATTATTAAAAAAATTCAATAGTAATTGCACTATTTTTGTTATACTTAAAAA
2976 TAGAAAAAAAGATTGATAGTTTTTACAAAACAGGTCAATTATTAGAGGAGAAAATATGGCTGCAGTATTAGAAAAAGAA
3055 AGACTTTTTTTCAAATTTATCAAAGTTTGGAAAAAGAATTACTAGAAATGACGGATTATATTCATTTTAGTGAAAATAA
3134 TTTAGTGTTTATTCTATAAAATTAGCAAATTTCATTCTTGGAGCAATTGTTGAATGTGAATCGTTACTAAAAGAATTA
3213 TTTAAACTGACAGAACACTATCAATCACTATCAGAAGATGAACAAAAAGAGTCATTAGAAAATAGTACTTATGTACAAG
3292 TAAATGCAGTATATAAACTTGATATGAAAACAATTTTTATGACATCAAAAAATTTTTTATTTTCAAGATATC
```
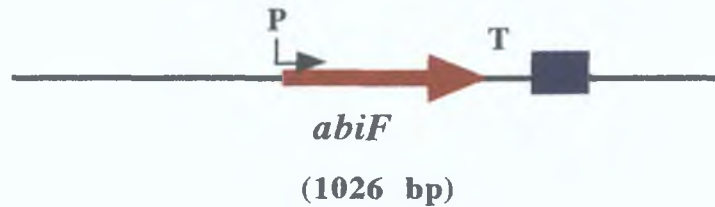
transcriptional terminator. The GC content of this gene is 26% which is exceptionally low for lactococcal genes which have an average of 37%; however, low GC contents appear to be a feature of lactococcal *abi* genes (see Chapter 3). Comparative analysis with known sequences in the EMBL and Genbank databases (release 82) using DNAstar (DNAstar Inc., Madison, WI) revealed no DNA sequence homology with any known sequence.

Interestingly, downstream of the phage resistance gene, an imperfect 23 bp sequence is directly repeated three and a half times (Fig. 4.2). This bears a strong resemblance to a feature found in the replication origin of many theta-replicating plasmids. All pCI305-type plasmids, for example, contain a conserved 22 bp sequence repeated three and a half times in the non-coding region of their minimal replicon (Hayes *et al.*, 1991). Hence this region could potentially encode a replication origin for pNP40. The 23 bp sequence of pCG1 is not homologous to the 22 bp direct repeat of the pCI305 family. It is worth noting that this putative origin is not located on the 7.6 kb *Eco*RI pNP40 fragment identified by Froseth *et al.* (1988b) as harbouring a plasmid origin.

Comparison of the deduced amino acid sequence of the 1026 bp ORF with known sequences in the Swiss and PIR and translated protein databases (release 82) using the DNAstar software programme (DNAstar Inc., Madison, WI) showed no significant similarity. No peptide motifs were identified from the Prosite database (release 11) using the MacPattern programme giving no insight as to the molecular basis of this mechanism. Analysis of this protein using the method of Kyte and Doolittle showed no regions of marked hydrophobicity leading us to propose a cytosolic location.

4.2.3 Localisation of phage resistance determinants on pNP40.

A restriction map of pNP40 was generated using a number of restriction enzymes chosen for their ability to restrict pNP40 infrequently (Fig. 4.3).

Hybridisation analyses, using the pNP40 fragments cloned in pPG01 (AbiE) and pPG23 as probes, localised this phage resistance gene and the AbiE determinants close to one another on pNP40 (Fig. 4.3). In fact, the original fragment encoding AbiE (pPG01) overlaps with the 5.6 kb *Eco*RV pNP40 fragment on pCG1, but subcloning of pCG1 has shown the overlapping region to be inessential for the pCG1-encoded resistance. *abiEii*, one of the ORF's responsible for the AbiE mechanism, encompasses 69 bp of this region. Additional hybridisation analyses using ISS1, IS981 and IS904 DNA as probes, revealed the presence of two copies of ISS1 and a single copy of IS981 on pNP40. No homology was detected to IS904. The approximate locations of these IS elements, and of the fragment identified by Froseth *et al.* (1988b) as harbouring the nisin resistance gene and the plasmid origin, are indicated in Fig. 4.3.

4.2.4 Resistance encoded by pCG1

A number of assays were performed to assess the nature of the resistance encoded by pCG1. Plaques assays on sensitive and resistant hosts indicated that the EOP of øc2 on either *L. lactis* ssp. *lactis* PG020 or PG023 was significantly diminished relative to plasmid-free *L. lactis* ssp. *lactis* MG1614 (Table 4.2). This decrease in EOP was accompanied by a reduction in plaque diameter from 3 mm to <1 mm. Resistance to ø712 was manifested by pinpoint plaques at an EOP of 3.0×10^{-6}. Neither ø712 nor øc2 plaqued on MG1614/pNP40. The following assays were performed to examine the type of resistance encoded by pCG1 to ø712 and øc2 and to compare it with pNP40-encoded resistance.

ø712: The presence of pCG1 did not affect the ability of ø712 to adsorb to *L. lactis* ssp. *lactis* MG1614. Over 95% of ø712 still adsorbed to PG020, demonstrating that the phage resistance gene acts post-adsorption (Table 4.3). To determine if R/M was involved in the observed phenotype, host-dependent plaquing efficiency was assessed. In the case of R/M systems, phage which become modified as a result of propagation on the resistant host, become susceptible to the resistance mechanism after

Table 4.2

Plaquing efficiencies of ø712 and øc2 on *L. lactis* ssp. *lactis* MG1614 and PG020

Phage	MG1614	PG020
ø712 MG1614	1 0	3 0x10^{-6}
ø712 MG1614 PG020	1 0	3 1x10^{-1}
ø712 MG1614 PG020 MG1614	1 0	3 4x10^{-1}
øc2 MG1614	1 0	8 0x10^{-4}
øc2 MG1614 PG020	1 0	5 0x10^{-1}
øc2 MG1614 PG020 MG1614	1 0	4 0x10^{-1}

Table 4.3

% Adsorption, % cell death, ECOI and burst size of ø712 and øc2 on *L. lactis* ssp. *lactis* MG1614, PG020 and MG1614/pNP40

Strain	Resistance	% adsorption	% cell death	ECOI	Burst size
<u>ø712</u>					
MG1614	none	97 0	100	1 0	54
PG020	AbiF	95 7	90	0 8	6
MG1614/pNP40	total	97 3	42	0 03	np
<u>øc2</u>					
MG1614	none	99 4	100	1 0	161
PG020	AbiF	99 1	94	0 77	13
MG1614/pNP40	total	98 6	10	0 0004	np

ECOI-Efficiency of centre of infection
np- not possible to evaluate

propagation on the sensitive host In this instance, ø712 became permanently modified as a result of propagation on PG020 and therefore did not rely upon host-encoded methylation for modification (Table 4 2)

Examination of the number of cells which survive infection is a critical indicator of whether the phage resistance mechanism is early-acting, e g adsorption inhibition or R/M, or late-acting, i e abortive infection No significant reduction in % cell death was observed for cells containing pCG1 compared to plasmid-free MG1614 indicating a late target of inhibition 90% of ø712-infected PG020 cells died as a result of infection as compared with 42% when pNP40 was present in the cell (Table 4 3)

ECOI assays, which indicate the number of infected cells which release viable phage progeny, were carried out on sensitive and resistant hosts Infection of the sensitive host incurs an ECOI of 1 0 by definition The ECOI of PG020 is marginally reduced to 0 8 for ø712 ø712 infection of MG1614/pNP40 resulted in an ECOI of 0 03, a consequence possibly of the activity of the two phage resistance mechanisms

The number of phage progeny released (burst size) from PG020 was 6/cell as opposed to 54 for the sensitive host It was not possible to evaluate the burst size for cells containing pNP40 because of the low ECOI (Table 4 3) Since adsorption inhibition and R/M have been eliminated as mechanisms of resistance for the pCG1-encoded gene and considering the response of PG020 (i e high cell death and reduced burst size), we defined the pCG1-encoded system as Abi

øc2. The response of cells containing pCG1 to øc2 were similar to the results obtained for ø712 Over 98% of øc2 adsorbed to PG020 (Table 4 3) R/M was also discounted as øc2 did not become modified by classical host-encoded modification (Table 4 2) The cell death, ECOI and burst size effects were similar to those for ø712 Small reductions were observed for % cell death (6%) and ECOI (0 23), while burst size was reduced dramatically from 161 to only 13 This confirms that the pCG1-encoded resistance operates against ø712 and øc2 by an Abi mechanism (Table 4 3) This mechanism was designated AbiF in keeping with the nomenclature suggested by

Coffey *et al* (1991) and the ORF on pPG23 was designated *abiF*

The effect of pNP40 on øc2 proliferation is more marked than that of pCG1 Cell survival was increased to 90% which is not accountable for by an Abi mechanism alone, suggesting the operation of additional phage resistance mechanism(s) on pNP40 against øc2, acting at an earlier stage in the lytic cycle (Table 4 3) ECOI data revealed that very few cells containing pNP40 release viable phage progeny (ECOI=0 0004) as compared with pCG1-containing cells (ECOI=0 77) This striking contrast strongly suggests that pNP40 encodes at least two phage resistance mechanisms against øc2 AbiF, plus an early-acting mechanism

4.2.5 Effect of pCG1 and pNP40 on øDNA replication

The phage DNA content of sensitive and resistant hosts was examined at 15 minute intervals following ø712 infection (Plate 4 1) Normal phage infection occurred in plasmid-free MG1614 with øDNA detected within the cell after 15 minutes The intracellular øDNA concentration increased over time until 60 minutes when a considerable reduction was observed This reduction reflected øDNA packaging and release of phage progeny In the cells containing AbiF, phage DNA entered the cells as normal but replication was significantly retarded relative to the plasmid-free host, suggesting that AbiF acts at the level of DNA replication This corroborates the phenotypic evidence which suggested that *abiF* encodes an abortive infection-type mechanism In MG1614/pNP40, øDNA replication was completely retarded with no increase in øDNA concentration despite normal DNA internalisation as visualised by DNA hybridisation (data not shown)

When the effect of AbiF on øc2 DNA replication was monitored over time, an identical phenomenon was observed (Plate 4 2) The increase in the øDNA content of cells following infection was considerably less in PG020 than in the sensitive host with a high intracellular øDNA concentration not detected until 30 minutes following infection as opposed to 10 minutes in the case of the sensitive host, supporting the view

Plate 4.1. DNA content of *L. lactis* ssp. *lactis* MG1614, PG020 and MG1614/pNP40 hosts following infection with ø712 Lanes 1 to 6 show the *Hind*III-digested DNA isolated from infected hosts at 0, 15, 30, 45, 60 and 75 minutes, respectively, following infection of MG1614, PG020 and MG1614/pNP40 (panels A, B and C, respectively)

A

1 2 3 4 5 6   1 2
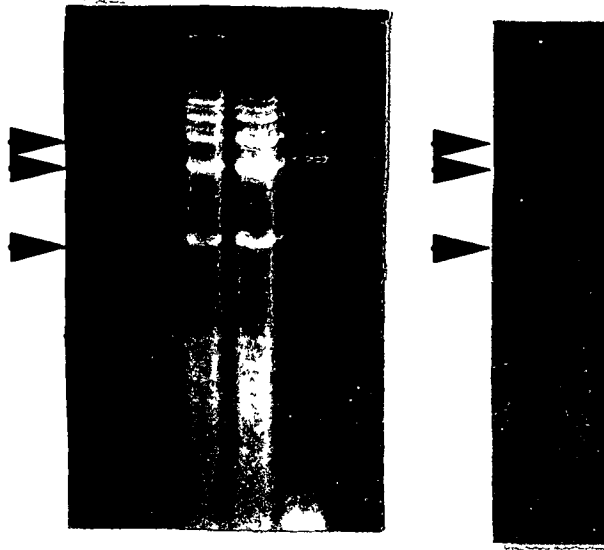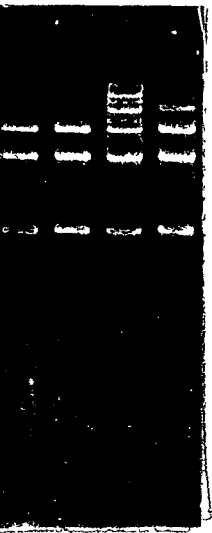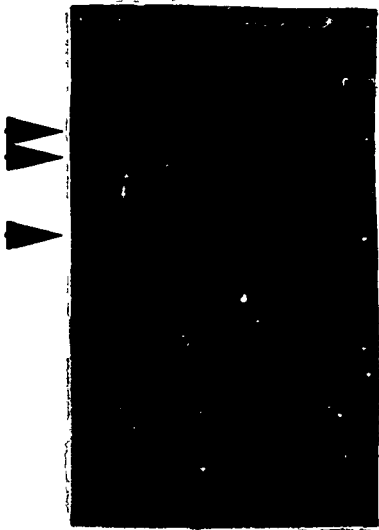
Plate 4.2.   Phage DNA content of *L lactis* ssp. *lactis* MG1614, PG020 and MG1614/pNP40 hosts following infection with øc2. Panels A, B and C represent MG1614, PG020 and MG1614/pNP40 respectively Lanes 1 to 6 show *Hind*III-digested DNA isolated from infected hosts at 0, 5, 10, 20, 40 and 60 minutes respectively following infection in the case of panel A and at 0, 5, 10, 30, 60 and 120 minutes respectively in the case of panels B and C
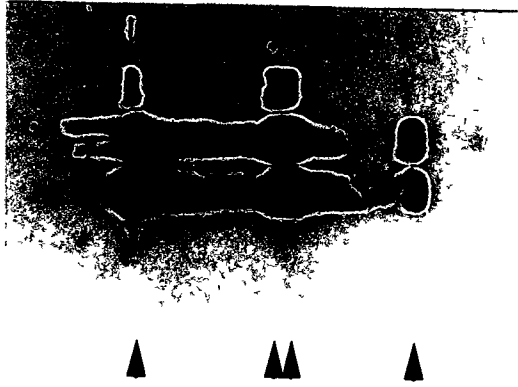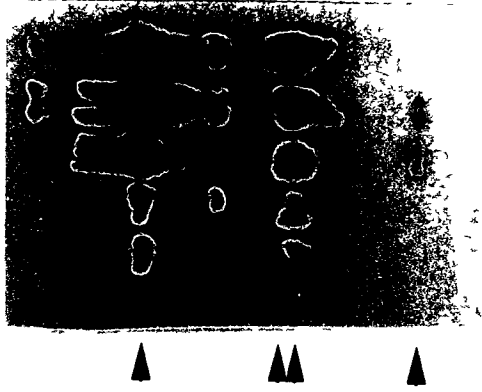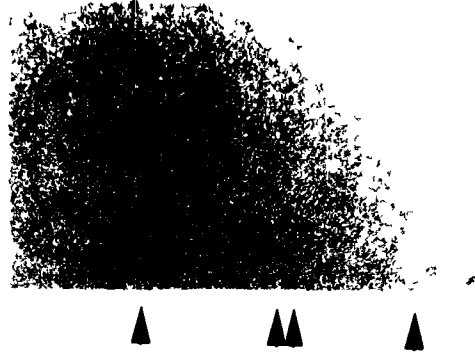
that *abiF* encoded a mechanism which depressed the rate of øDNA replication. A remarkable difference, however, was observed in cells containing pNP40. Phage c2 DNA could not be detected in the cell until 30 minutes after infection and then, the concentration was comparatively low. This delay or inefficiency in phage DNA internalisation supports earlier evidence which suggested that an early-acting mechanism against øc2 is operational in cells containing pNP40.

4.3 DISCUSSION

The resistance encoded by pCG1 bears the phenotypic hallmarks of an abortive infection-type mechanism. The EOP and plaque size were reduced for both prolate- and isometric-headed phages on strains containing pCG1. We have shown that phage adsorbed normally to PG020, their DNA was internalised but phage DNA replication was significantly retarded. This seemingly modest effect of lowering the number of phage genomes generated within the cell can result in reduced production of phage gene products (Moineau *et al.*, 1992). In this case, the outcome was a considerable reduction in the number of phage progeny produced by infected PG020 cells, and even failure of some of the cells (20-23%) to give rise to any progeny.

AbiF co-exists with a previously identified phage resistance mechanism, AbiE, on pNP40 (Chapter 3). Examination of the effect of AbiE on ø712 DNA proliferation showed that it also encoded abortive infection but acted late in the lytic cycle, apparently after phage DNA replication had taken place, presumably at the level of transcription/translation or phage packaging/release (Chapter 3). The combination of two mechanisms acting at consecutive stages in the lytic cycle permitted only 3% of infected cells to produce progeny and reduced the burst size to undetectable levels.

The mechanism encoded by *abiF* appears to act at the level of phage DNA replication. In lactococci, AbiA (pTR2030) has also been shown to act at this stage in

the lytic cycle (Hill *et al* , 1991a) and in *E coli*, when the ColV plasmid is present in a host, no phage DNA replication takes place (Reakes *et al* , 1987) The molecular basis of these systems is unknown although in the case of AbiA (pTR2030), it has been shown to be phage *ori* specific A novel phage resistance mechanism, generated by Hill *et al* (1990a) which was mediated by a recombinant plasmid harbouring a cloned ø50 origin was found to mimic this effect The authors suggested that the multi-copy plasmid carrying the phage *ori* titrated factors involved in phage DNA replication away from the true phage *ori*, thus reducing the number of phage genomes generated As the phage resistance encoded by pCG1 appeared to involve a protein product, DNA titration of phage replication factors did not explain this mechanism

AbiF is encoded by a single ORF which encodes a 41 2 kDa protein To assess if competive binding to the phage origin by the gene product is a possibility, we examined the deduced aa sequence of *abiF* for potential DNA-binding motifs None were detected using the MacPattern programme, although this does not eliminate binding of phage DNA as a potential mechanism, as these programmes are not infallible It is possible that the *abi* gene product affects the activity of a phage gene product The increasing knowledge of the molecular biology of phage genomes which is now becoming available should help establish the molecular basis of this Abi mechanism

Downstream of *abiF*, a 23 bp sequence which is repeated three and a half times was identified This interesting motif bears a strong resemblance to the genetic organisation of the plasmid origin of many theta-replicating plasmids The lactococcal pCI305-type replicons, for example, contain a conserved 22 bp repeat repeated three and a half times (Hayes *et al* , 1991) While the 23 bps repeat sequence exhibited no sequence homology to this 22 bp sequence, nevertheless, this region could potentially code for a replication origin for pNP40 Intriguingly, this region does not correspond to the 7 6 kb *Eco*RI fragment of pNP40 identified by Froseth *et al* (1988a) as encoding the pNP40 origin of replication which was exploited by Froseth and McKay (1991a) in

the construction of a food-grade vector for *Lactococcus* This observation, in addition to the fact that pNP40 encodes two copies of ISS1, suggests that the plasmid may have originated as a consequence of a cointegration event, possibly during conjugative transfer, as pNP40 is also self-transmissable Cloning of overlapping fragments from pNP40 into a replication probe vector should establish if this region encodes an operational plasmid origin

pNP40 is unique in that it is the only lactococcal phage resistance plasmid isolated to date on which two distinct Abi mechanisms have been identified In fact, the determinants for AbiE and AbiF lie in close proximity to one another on pNP40 Analyses of many other abortive infection plasmids have shown that they encode additional mechanisms pTR2030 (Hill *et al* , 1989a), pTN20 (Durmaz *et al* , 1992), pKR223 (Laible *et al* 1987) and pBF61 (Froseth *et al* , 1988b) were all found to encode R/M in addition to Abi and in the case of pCI528, Abi was found in association with adsorption inhibition (Costello, 1988)

The resistance encoded by pNP40 against øc2 cannot be accounted for through the action of AbiF alone The presence of pNP40 in the host strain confers a survival potential on the cell considerably in excess of that which could be bestowed by a late-acting mechanism such as Abi Furthermore, there is a tremendous reduction in the number of infected cells releasing viable phage, only 0 04% as compared to 77% in cells containing AbiF alone Finally, no DNA is detected within cells containing pNP40 until 30 minutes following infection as compared to 5 minutes in the sensitive host or hosts containing AbiF This indicated the operation of an additional early-acting mechanism on pNP40 against øc2 making pNP40 the first lactococcal plasmid which encodes three phage resistance determinants Chapter 5 describes further analysis of this mechanism

CHAPTER V

A NOVEL DNA INJECTION BLOCKING MECHANISM MEDIATED
BY THE LACTOCOCCAL BACTERIOPHAGE RESISTANCE PLASMID
pNP40

5.1 INTRODUCTION

It has previously been demonstrated that AbiF-mediated resistance alone could not account for the total insensitivity to øc2 exhibited by pNP40-containing cells (Chapter 4) and that a second mechanism of resistance on pNP40 was active against øc2 which operated at an early stage in the lytic cycle. Recent studies on the lytic cycle of phage have shown that there are potentially many mechanisms by which cells may inhibit early phage infection (for review see Klaenhammer and Fitzgerald, 1994). The most comprehensive study of early phage infection in lactococci concerned the adsorption by phage øc2 (and six other phage) to *Lactococcus lactis* ssp *lactis* C2 (Valyasevi *et al*, 1991, Valyasevi *et al*, 1994, Geller *et al*, 1993 and Monteville *et al*, 1994). Carbohydrate moieties in the cell wall and specific membrane proteins, PIP (phage infection protein) and an un-named 32 kDa protein, were found to be involved in phage adsorption and DNA injection (Monteville *et al*, 1994). Following DNA internalisation (unless digested by restriction endonucleases), the phage initiates events which result in the death of the host (Powell *et al*, 1992). Any mechanism which terminates the phage infection prior to this stage increases host survival and is regarded as an early-acting mechanism.

To date, only two early-acting phage resistance mechanisms are recognised in lactococci classical adsorption blocking, and restriction/modification (R/M). A number of plasmids have been identified which encode adsorption inhibition. Two of these, pCI528 (Lucey *et al*, 1992) and pSK11 (Sijtsma *et al*, 1990), have been shown to direct the production of exopolysaccharides which mask the phage receptors R/M systems comprise a restriction endonuclease, which degrades the phage DNA after it is injected into the host, and a modification component which protects the host DNA from cleavage. They are widely distributed among the lactococci and their determinants have been located to both plasmid and chromosomal DNA (Klaenhammer and Fitzgerald, 1994)

This study compares the early stages of phage øc2 infection of plasmid-free *L lactis* ssp *lactis* MG1614 and MG1614 containing pNP40 in an effort to understand the early-acting mechanism by which pNP40 prevents øc2 proliferation

5.2 RESULTS

5.2.1 Adsorption of øc2 to pNP40-containing cells

It has been demonstrated previously that *abi*F mediated resistance alone cannot account for the total insensitivity to øc2 exhibited by pNP40-containing cells Thus, it is likely that pNP40 encodes an additional phage resistance mechanism against this phage which operates at a stage early in the lytic cycle (Chapter 4) A number of assays were conducted to assess if the enhanced level of pNP40-encoded resistance against øc2 is mediated by adsorption blocking or by R/M, the only early-acting mechanisms recognised in lactococci to date

Adsorption assays using *L lactis* spp *lactis* MG1614 and MG1614 containing pNP40 revealed that over 98% of øc2 particles adsorbed to both hosts, indicating that the early-acting mechanism did not operate by classical adsorption blocking

The possibility that non-specific adsorption was occurring was discounted by examining the ability of these hosts to adsorb phage øc2 irreversibly The addition of L-rhamnose has previously been shown to desorb phage which have only undergone the initial attachment step (Valyasevi *et al*, 1991) We confirmed that in the very early stage of infection, L-rhamnose can indeed desorb bound phage øc2 from both plasmid-free MG1614 and MG1614/pNP40 However, as time proceeded, the ability of L-rhamnose to desorb øc2 was reduced until at 15 min, over 90% of phage were adsorbed irreversibly (Fig 5 1) This was observed for both MG1614 and MG1614 /pNP40, thus reaffirming that pNP40 does not encode a mechanism which prevents normal adsorption of phage to the cell wall

MG1614
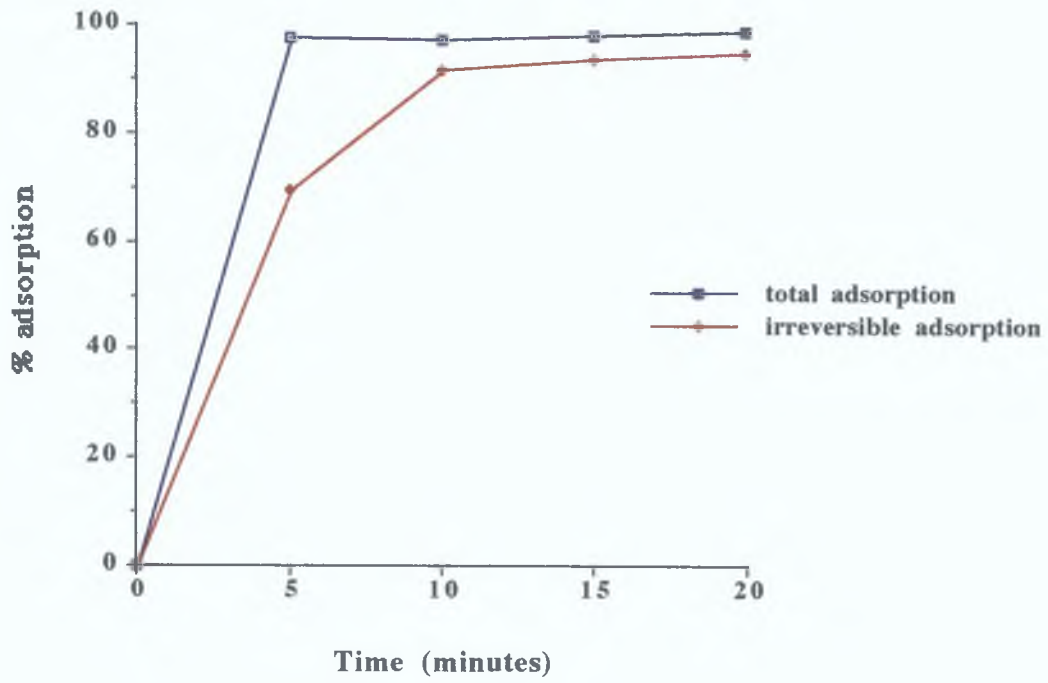
MG1614/pNP40
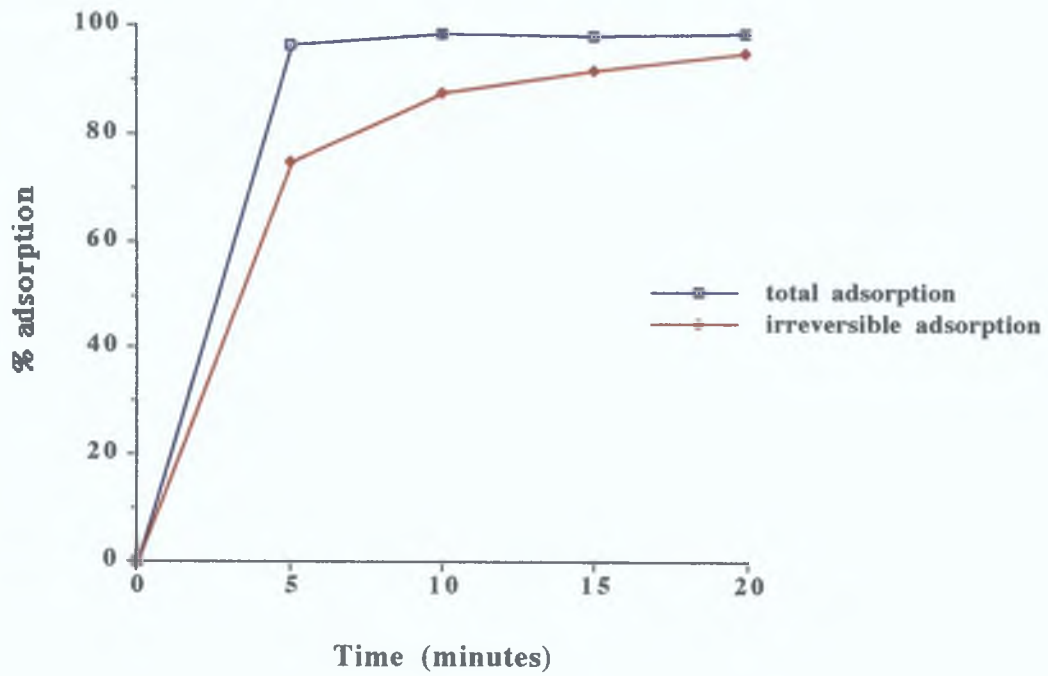
When øc2 infection of *L lactis* ssp *lactis* MG1614 and MG1614/pNP40 was examined using electron microscopy, the phage particles were shown to adsorb in a tail-first orientation to both hosts This further confirmed that pNP40 did not operate by interfering with phage binding to the host and that the adsorption assay results did not conceal non-specific adsorption by the phage (Plate 5 1)

5.2.2 Elimination of R/M involvement in the pNP40-encoded resistance

R/M is a second early-acting phage resistance mechanism commonly encountered in lactococci (for review, see Klaenhammer and Fitzgerald, 1994) It was important to eliminate it as the agent of the early-acting resistance encoded by pNP40, in particular since R/M and Abi systems can frequently be located on the same plasmid, e g pTR2030 (Hill *et al*, 1989a) As classical R/M studies require phage that are capable of plaquing on the resistant host, this presented difficulties as øc2 was incapable of plaquing on MG1614/pNP40 Furthermore, a mutant phage, møc2, chosen for its ability to overcome AbiF, and was therefore only be inhibited by the putative early mechanism, was also unable to plaque on MG1614/pNP40

It is unlikely, based on the data available for lactococcal R/M systems, that a putative pNP40-encoded R/M system alone could confer the level of resistance mediated by pNP40 to møc2 (EOP$<10^{-8}$) However, in previous studies systems have been overlooked, particularly when candidate phage were unavailable for R/M studies, e g pTR2030 (Hill *et al* , 1989a) and pKR223 (Laible *et al* , 1987) Therefore, as neither øc2 nor møc2 were suitable for R/M studies, two alternative phage-host systems were used to assess the possibility of pNP40 encoding an R/M system Phage ødrc3 has been shown by plaque assay to propagate efficiently (EOP of 1 0) on the original pNP40-containing host *L lactis* ssp *lactis* biovar *diacetylactis* DRC3 This phage is therefore capable of circumventing the resistance mechanisms encoded by pNP40 Propagation of ødrc3 on its alternative host, *L lactis* ssp *lactis* biovar *diacetylactis* 18-16, resulted in phage which plaqued with equal efficiency on DRC3 and 18-16,

Plate 5.1. Electron micrographs of phage øc2 adsorption to *L. lactis* ssp. *lactis* MG1614 (A) and MG1614/pNP40 (B) hosts.
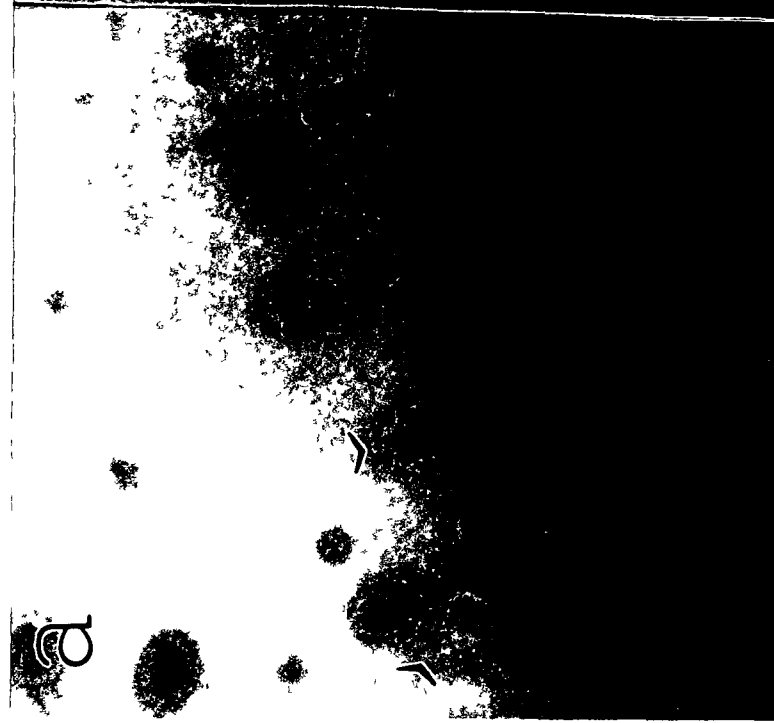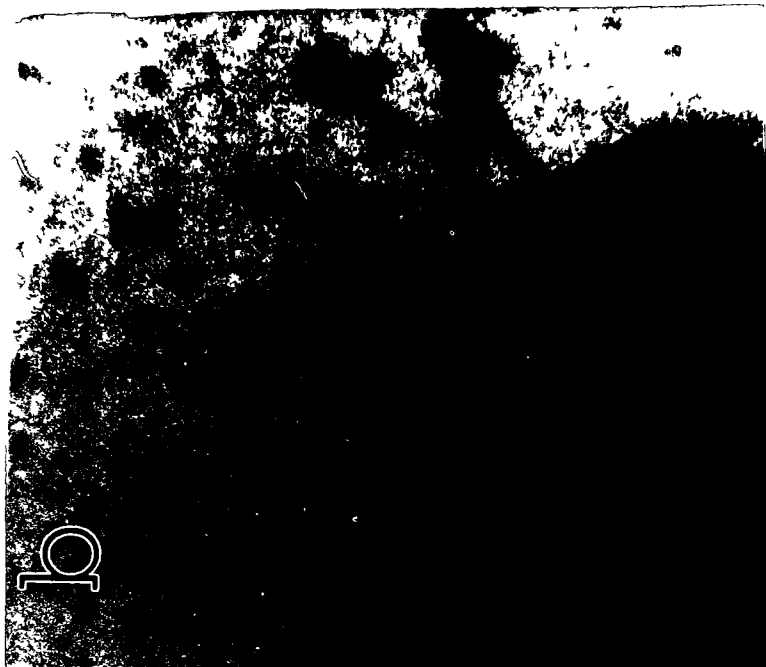
suggesting that a reversible modification was not the basis of the resistance of this phage to the pNP40-directed mechanisms Plasmid profile and hybridisation analysis confirmed that pNP40 was not present in host 18-16 (data not shown) Similarly, ø3, propagated on strain *L lactis* ssp *lactis* biovar *diacetylactis* DPC220 at 21°C, plaqued with an EOP of 1 0 on DPC220 and DPC220(pNP40) (Harrington and Hill, 1991) Phage ø3, like phage ødrc3 was capable, therefore, of overcoming the resistance mechanisms encoded by pNP40 regardless of the previous host, and did not rely upon classical host-encoded modification for this resistance Thus, despite investigation in three different hosts using three different phage, no evidence of R/M was detected

5.2 3 Electroporation circumvents the early-acting mechanism

Previously, the phage DNA content of øc2-infected MG1614 and MG1614/ pNP40 cells was exammed at time intervals following infection (Chapter 4) No phage DNA was detected within pNP40-containing cells until 30 minutes after infection as compared to only 5 minutes in the sensitive host Furthermore, even at 30 minutes, the DNA concentration was very low suggesting that the mechanism operates prior to DNA entry into the cell

Electrotransformation of øc2 DNA into pNP40-containing cells effectively permits the phage to bypass the adsorption and DNA injection stages of infection To investigate if the putative early-acting mechanism could be circumvented in this manner, the ECOI of øc2 following electroporation was compared with the ECOI determined following a conventional infection (Table 5 1) The ECOI for øc2 on MG1614/pNP40 differed by approximately three log cycles following phage DNA electroporation as compared to a conventional infection This suggests, since adsorption blocking was discounted, that pNP40-mediated resistance to øc2 operates at two levels, i e by blocking phage DNA penetration into the cell and by aborting phage DNA replication through the AbiF-mediated system

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Table 5.1

ECOI of phage c2 on *L. lactis* ssp. *lactis* MG1614, PG020 and MG1614/pNP40 following

conventional infection or after electroporation of phage øc2 DNA

Strain	Normal ECOI	ECOI after electroporation
MG1614	1 0	1 0
PG020 (AbɪF)	0 77	0 26
MG1614/pNP40	0 0004	0 11

5.3 DISCUSSION

Three categories of naturally occurring phage resistance mechanisms are recognised in lactococci: adsorption inhibition, R/M and abortive infection (Klaenhammer and Fitzgerald, 1994). Based on the results of this study, we propose a fourth category to account for the enhanced resistance (relative to *abi*F alone) mediated by the lactococcal plasmid pNP40 against øc2 which does not conform to the criteria defining any of these mechanisms.

The results presented here indicate that the lactococcal plasmid pNP40 encodes an early-acting phage resistance mechanism but no evidence of either classical adsorption blocking or R/M could be detected. The proposal that pNP40 encodes a novel injection blocking mechanism is supported by several lines of evidence.

(i) Phage øc2 particles adsorbed to sensitive hosts and to MG1614/pNP40 with equal efficiency, and electron microscopy showed that this attachment occurred in the normal tail-first orientation. This process was initially reversible by the addition of L-rhamnose and, over time, became irreversible, demonstrating that the adsorption function of the host cell wall was not impaired and that resistance occurred at a subsequent step in the infection process.

(ii) Only 10% of cells died as a result of infection (Chapter 4), suggesting that this mechanism must operate prior to corruption of host functions or DNA degradation by the infecting phage.

(iii) Internalisation of the genomes of the infecting phage was delayed as evidenced by the inability to detect bacteriophage-specific DNA within infected pNP40-containing cells until at least 30 minutes following infection as opposed to 5 minutes in the case of a sensitive host (Chapter 4).

(iv) A significant increase in ECOI was detected following circumvention of the early stages of infection, by electroporation of phage DNA into resistant hosts, also supporting the view that the early-acting pNP40-encoded resistance acts at the level of

phage DNA penetration into the cell

Monteville *et al*, (1994) have shown that in the case of øc2 infection, a membrane protein PIP (phage infection protein) is essential for phage interaction with the host cell membrane, and that a second 32 kDa membrane protein may also play a role in phage infection or translocation of phage DNA across the membrane It is likely, based on the results presented here, that it is an alteration at the level of the cell membrane that prevents phage DNA injection into the pNP40-containing host Several models can be envisaged to accommodate this hypothesis a protein product, encoded by pNP40, could prevent production of either the MG1614 PIP or the 32 kDa protein equivalents, this putative product could prevent insertion of either of these proteins into the cell membrane by competing for sites or by interacting with these components in such a way so as to prevent insertion into the membrane, or the pNP40-encoded product could, by interaction with either of the membrane-located proteins, prevent the involvement of these proteins in phage DNA ejection from the phage head and translocation of the DNA across the membrane

The early-acting pNP40-encoded resistance mechanism is not active against the small isometric-headed phage ø712 since phage DNA could be detected within pNP40-containing cells within 15 minutes following ø712 infection (Chapter 4) This supports evidence by Valyasevi *et al* (1991) that all phages do not have the same infection requirements with respect to the host cell membrane The inferior resistance mediated by pNP40 to øc2 at elevated temperatures (37°C) supports the concept of a mechanism operating at the level of the cell membrane whose fluidity at this temperature could significantly alter any interaction between the pNP40-encoded protein and the membrane components required for øc2 infection It seems likely therefore, that at least four categories of phage resistance are operative in lactococci against phage adsorption blocking, R/M, Abi and a mechanism which inhibits phage DNA penetration

CHAPTER VI

CLONING AND DNA SEQUENCE ANALYSIS OF A PLASMID-
ENCODED *RecA* HOMOLOGUE FROM THE LACTOCOCCAL PHAGE
RESISTANCE PLASMID pNP40: A ROLE FOR RECA IN ABORTIVE
INFECTION

6.1 INTRODUCTION

RecA is a multifunctional enzyme which plays a pivotal role in homologous recombination and in the SOS response in *Escherichia coli* (for reviews see Miller and Kokjohn, 1990 and Radding, 1991) It has the ability to bind single-stranded and double-stranded DNA simultaneously which promotes recombination between homologous DNA's with concomitant hydrolysis of ATP Furthermore, in response to DNA damage, it acts as a co-protease in the cleavage of the LexA repressor which results in derepression of a group of DNA repair proteins known as the SOS regulon RecA is also required for prophage induction in several bacteria, for example, the cI gene product of the λ prophage, which acts as a repressor of prophage induction, is cleaved in the presence of the *E coli* RecA More recently, Duwat and Gruss (1994) have proposed additional functions for RecA in response to oxygen and thermal stress in lactococci, suggesting that it has a general role in the regulation of genes associated with different types of stress Furthermore, in *Vibrio cholerae*, RecA has been implicated in bacteriophage resistance where it was discovered that the *sulA* gene product, which contributes to an abortive infection mechanism, is cleaved by RecA (Biswas *et al* ,1992) Of added interest is the suggestion that LexA may be involved in regulation of an abortive infection gene in *Escherichia coli* (Gupta and McCorquodale, 1988)

Until recently, comparatively little was known about RecA in gram positive bacteria However, the DNA sequences of the *recA* genes from several gram positive species have now been elucidated, including two from *Lactococcus lactis* ssp *lactis* and one from *L lactis* ssp *cremoris* (Duwat *et al* 1992) The amino acid sequences of all three lactococcal RecA's were identical, despite minor differences at the DNA level, and were 61% and 56% identical to the RecA's of *Bacillus subtilis* and *E coli*, respectively In all investigated gram negative and gram positive bacteria, the *recA* gene has been located to the chromosome In this study, an open reading frame has been identified on

the lactococcal plasmid pNP40 which codes for a RecA-like protein

The identification of three mechanisms of phage resistance encoded by pNP40 are described in Chapters 3, 4 and 5 of this thesis The determinants for two of these phage resistance mechanisms, AbiE and AbiF, have been analysed at the DNA sequence level and lie in close proximity This study describes DNA sequence analysis of the intervening region which revealed the presence of two open reading frames (ORF's) The deduced amino acid sequence of one of these ORF's codes for a RecA-like protein

6.2 RESULTS

6.2.1 DNA sequence analysis

Sequence analysis of 3 3 kb of DNA located between the determinants for AbiE and AbiF revealed the presence of two ORF's which were found to be transcribed in the reverse orientation relative to the phage resistance genes (Fig 6 1) One of these ORF's was found to code for a recA homologue based on DNA sequence homology It is 1023 bp in length and has the capacity to encode a protein of 341 amino acids with a predicted molecular mass of 37 2 kDa (Fig 6 2, second ORF) This corresponds favourably with the size of known RecA proteins A putative RBS (GAAAGGAG) with a ΔG value of -16 2 kcal/mole complementary to the 16S rRNA of gram positive and gram negative bacteria was found 6 bp upstream of the ATG start codon A putative -10 sequence (AATAAT) was identified 99 bp upstream of the RBS separated by 19 bp from a -35 sequence (TTGTAG) each of which partially resembled the -10 and -35 sequences (TATAAT and TTGACA, respectively) of the consensus lactococcal promoter (van de Guchte et al , 1992) However, no TG dinucleotide was located upstream of the -10 sequence as has been identified in the promoters of many lactococcal genes (de Vos, 1987) A 14 bp inverted repeat with a ΔG value of -25 2

90

Figure 6.1. Genetic organisation of the 7.2 kb region of pNP40 showing the determinants for AbiE and AbiF, RecA$_{LP}$ and ORFU. Direction of the arrows denotes direction of transcription The locations of putative promoters and terminators are indicated

ΔG=-25.2 kcal/mole

```
A    T
A    T
A    T
T    A
C    G
A    C
G    C
G    C
G    C
A    T
A    T
A    T
A    T
```

abiEi   *abiEii*   *recA*(LP)

(861 bp) (894 bp)   (1023 bp)

P
└→ denotes putative promoter

ΔG=-13.4 kcal/mole

ORFU
(1464 bp)

abiF
(1026 bp)
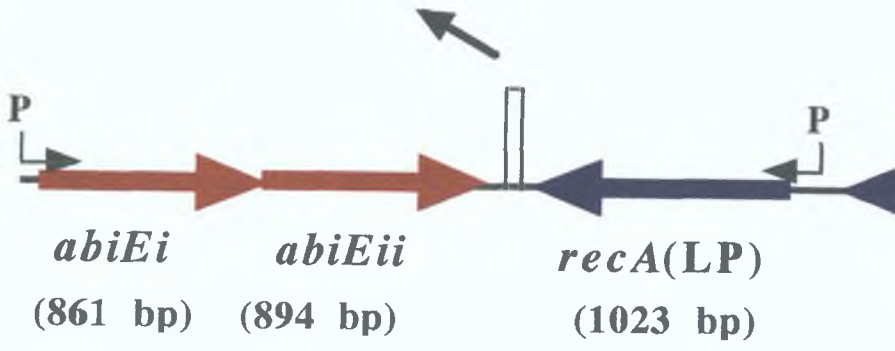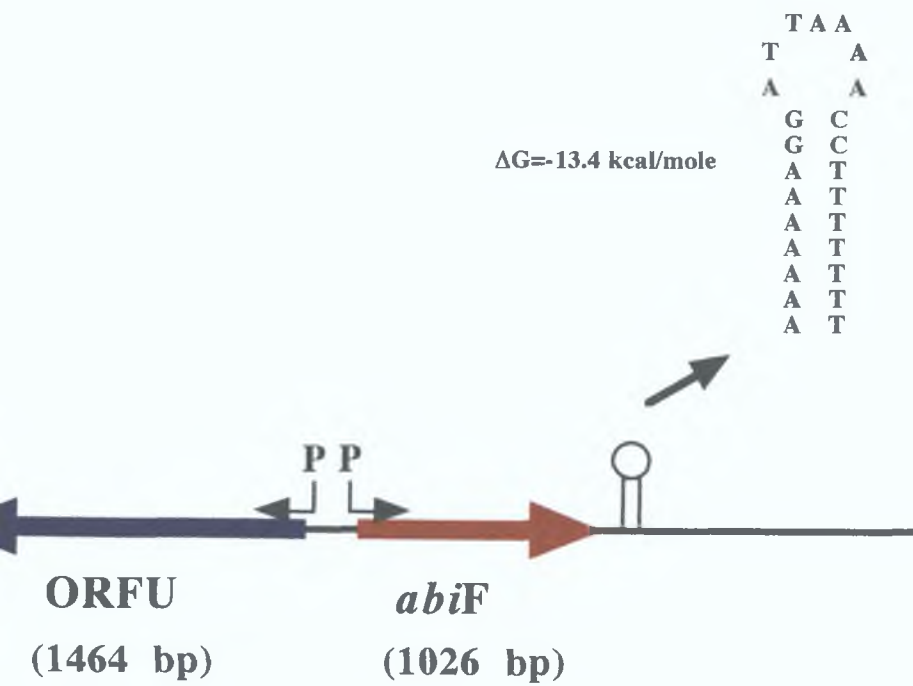
Figure 6.2. DNA sequence of 3.3 kb region of pNP40. The ammo acid sequences of ORFU and RecA$_{LP}$ are specified by three letter code designation Putative RBSs, -10 and -35 sequences and transcriptional terminator are underlined * denotes start of putative LexA binding site

1   AGTAACCTTTACTACACTATTTTATTGTAAAAAAAAAGTCTTTC

45 CATGATGGCGCTTGTTTTAAAAACAGAGGCGTGGGAAGTACTGATATATATATTAAAACATATTTTAGTTTGAAATTCA
                                                                        *
124 AGAGATTTTAATTAAAAATAGTTTTTTTTTTACTGGAAAAGTTTAAATTCCAAACGTTTTACTATTTATTCTGATATTAT
                                *   *
203 ATATTGATTTTAGGACTTTTTCAATGTATAATATCATTTAAACAGATATTTTGTGTTTGATATACTACTGGAGGTGCAA
    ‾‾‾‾‾‾‾‾   ‾‾‾‾‾‾‾‾‾‾   ‾‾‾‾‾‾
      -35   * -10   RBB

       Met Gly Ile Gln Ile Leu Asn Asn Gln Phe Asp Tyr Ser Leu Glu Pro Arg Arg Ala Ile   20
282 ATG GGA ATA CAA ATA CTA AAT AAC CAA TTT GAC TAT TCA CTT GAA CCT CGT CGA GCT ATC

       Phe Phe Glu Asp Val Lys Ser Asn Tyr Ala Ser Ile Glu Cys Ile Glu Arg Gly Leu Asn   40
343 TTT TTT GAA GAT GTT AAA TCT AAT TAC GCT TCA ATT GAA TGT ATT GAA CGT GGG TTA AAT

       Pro Leu Thr Thr Ser Leu Cys Val Met Ser Arg Ala Asp Asn Ser Asn Gly Leu Thr Leu   60
403 CCC CTG ACT ACT TCT CTT TGT GTA ATG AGT AGA GCT GAT AAT TCA AAT GGC TTA ACA CTT

       Ala Ala Ser Pro Thr Phe Lys Lys Val Phe Gly Met Ser Asn Val Ser His Ser Lys Glu   80
463 GCT GCT AGT CCA ACT TTC AAG AAA GTA TTT GGA ATG TCT AAT GTT AGT CAT TCC AAA GAA

       Leu Pro Phe Leu Val His Asn Arg Lys Phe Asn Tyr Arg Leu Trp Tyr Lys Lys His Thr   100
523 CTT CCG TTC CTG GTA CAT AAC CGT AAA TTT AAC TAT CGG CTA TGG TAC AAA AAA CAT ACA

       Asp Ile Phe Gly Gln Thr Val Glu Pro Asp Pro Lys Tyr Ile Ser Glu Val Glu Arg Trp   120
583 GAT ATT TTT GGA CAG ACT GTA GAA CCT GAT CCA AAA TAT ATT TCT GAA GTT GAA CGT TGG

       Ala Arg Gln Thr Tyr Ile Val Pro Pro Gln Met Leu Leu Tyr Ile Lys Lys Asn Leu Glu   140
643 GCA AGA CAA ACT TAT ATT GTT CCT CCT CAA ATG CTG CTA TAT ATC AAA AAA AAT TTA GAA

       Val Ile Asn Ile Leu Arg Glu Ile Thr Ser Ile Asp Glu Ile His Ala Tyr Ser Ile Asp   160
703 GTA ATC AAT ATT TTG AGA GAA ATT ACC TCT ATA GAT GAA ATC CAT GCT TAC TCT ATA GAT

       Glu Ser Cys Leu Asp Val Thr Glu Ser Leu Asp Phe Phe Phe Pro Glu Ile Thr Asn Thr   180
763 GAA TCC TGT TTA GAT GTT ACC GAA TCT TTG GAC TTC TTC TTT CCT GAA ATT ACT AAT ACA

       Tyr Glu Gln Met Asp Lys Leu Ala Gln Met Leu Gln Arg Lys Ile Tyr His Lys Thr Gly   200
823 TAC GAA CAA ATG GAT AAG TTA GCT CAA ATG CTG CAG CGT AAA ATT TAT CAT AAA ACT GGC

       Leu Tyr Val Thr Ile Gly Met Gly Asp Asn Pro Leu Leu Ala Lys Leu Ala Met Asp Asn   220
883 TTA TAT GTG ACA ATT GGA ATG GGA GAC AAT CCA CTT CTC GCA AAA CTT GCA ATG GAT AAT

       Tyr Ala Lys His Asn Thr Asn Met Arg Ala Leu Ile Arg Tyr Glu Asp Val Pro Ser Lys   240
943 TAT GCT AAA CAT AAT ACC AAC ATG AGA GCC TTG ATT CGC TAT GAA GAT GTT CCC TCT AAG

       Val Trp Ser Ile Ser Asp Met Thr Asp Phe Trp Gly Ile Asn Val Arg Thr Glu Ala Arg   260
1003 GTG TGG TCA ATC TCT GAT ATG ACT GAC TTT TGG GGT ATT AAT GTA AGA ACT GAA GCA CGT

       Leu Asn Lys Leu Gly Ile His Ser Ile Lys Glu Leu Ala His Ala Asp Pro Asp Met Leu   280
1063 TTA AAT AAG TTG GGG ATT CAT TCA ATA AAA GAG CTT GCT CAT GCT GAC CCT GAT ATG TTA

       Lys Arg Glu Leu Gly Val Ile Gly Leu Gln Gln Phe Phe His Ala Asn Gly Ile Asp Glu   300
1123 AAG CGT GAA TTA GGA GTG ATT GGG CTT CAA CAA TTT TTT CAT GCT AAC GGA ATT GAT GAA

       Thr Arg Leu Thr Asp Lys Tyr Lys Arg Lys Ser Val Ser Phe Ser Asn Ser Gln Thr Leu   320
1183 ACA CGT TTA ACT GAC AAG TAT AAA AGG AAA TCT GTC AGT TTC TCA AAT AGT CAA ACC CTA

       Pro Arg Asp Tyr Thr Arg Lys Ser Glu Ile Gly Leu Ile Ile Asn Glu Met Ala Glu Gln   340
1243 CCT AGA GAT TAT ACA CGT AAA TCG GAA ATA GGA TTG ATA ATA AAT GAA ATG GCT GAA CAA

       Val Ala Val Arg Leu Arg Lys Ser Lys Lys Lys Ala Thr Asn Phe Ser Leu Phe Val Gly   360
1303 GTT GCT GTG AGA CTA AGA AAA TCA AAG AAA AAA GCG ACA AAT TTT TCG CTC TTT GTG GGA

       Phe Ser Met Ala Asp Tyr Lys Lys Ser Leu Ser Val Ser Arg Lys Ile Glu Pro Thr Ser   380
1363 TTT TCA ATG GCT GAT TAT AAA AAG TCA CTC TCA GTT TCT AGA AAG ATT GAA CCA ACT AGC

       Ser Thr Lys Asp Leu Gln Glu Ile Ala Thr Arg Leu Phe Asn Glu Lys Tyr Asp Glu Gly   400
1423 TCT ACT AAA GAT TTA CAA GAA ATT GCT ACC AGA CTA TTT AAT GAA AAA TAT GAT GAA GGC

       Ala Val Arg Arg Ile Gly Val Ser Ala Asn Asn Leu Ile Asp Glu Pro Tyr Gln Leu Ile   420
1483 GCA GTT AGA CGG ATT GGG GTT AGT GCC AAT AAT CTG ATA GAC GAA CCT TAT CAA CTT ATT

       Ser Leu Phe Asp Ser Asp Glu Glu Asn Glu Glu Thr Ile Lys Gln Lys Lys Asp Glu Ala   440
1543 TCA CTC TTT GAT TCT GAT GAA GAA AAC GAA GAA ACA ATT AAA CAA AAA AAG GAC GAA GCT

       Val Gln Glu Ala Leu Asp Ser Ile Arg Gln Lys Tyr His Phe Val Ser Val Gln Lys Ala   460
1603 GTG CAA GAA GCA CTT GAC TCG ATT CGT CAA AAA TAT CAC TTT GTT TCT GTT CAA AAA GCA

       Thr Val Leu Lys Lys Gly Ser Arg Ala Val Ala Arg Ser Lys Met Val Gly Gly His Ser   480
1663 ACT GTT CTT AAG AAA GGG TCA CGT GCA GTT GCA AGA AGC AAA ATG GTA GGA GGA CAC TCT

       Ala Gly Gly Leu Glu Gly Leu Asn Stop   488
1723 GCA GGT GGA TTG GAG GGC TTG AAT TGA GTAGTGTTGACAGGTCTTATAGTAAATATGAATCAATAAGAAC

1793 GTATGAAGATAGAGGGAAAATGAAATGAATCCTTTTGCGACTCCGAACTTCTTCAGCTCATCGTGATTATCATAAAGAA

1872 TTTACCTTTGAAGAGCCAGATTTTTCTTTGGAACAAGATGAGATTCTTACCATGATTTCTTTCGCAAAGAGCCTCAGCT

1951 TGAATTAATATAGAGTACCAGGTAGGTAAGAATTTAAAAAATGTAGATGGAATCATTGTAGATTGGAAAGATAAAACAG
                                                        ‾‾‾‾‾‾
                                                         -35
2030 GAATAATCCTAAAAAAAATAGATGGCCATTATTTAGAACTGGAATTTAGCTGCATCGTTAAAATTAACTGATAAACAAT
     ‾‾‾‾
     -10
2109 ATTTTGATTCCTACCTCAAACAATATTGAAAGGAGAAATCC
                                ‾‾‾‾‾‾‾‾‾
                                   RBS

       Met Glu Gln Pro Gln Tyr Asn Ser Tyr Lys Val Arg Lys Leu Asp Asp Pro Glu Glu Lys   20
2149 ATG GAA CAA CCT CAA TAT AAT TCT TAT AAA GTA AGA AAA TTA GAT GAT CCA GAA GAA AAG

       Lys Leu Ala Ile Leu Lys Ala Thr Gln Ser Ile Glu Lys Lys Phe Gly Ser Asn Thr Ile   40
2210 AAA TTA GCT ATT CTC AAA GCA ACA CAA TCG ATT GAA AAA AAG TTT GGC TCT AAT ACC ATT

       Leu Asn Glu Glu Gly Lys Ala Ser Gln His Val Gln Ala Leu Pro Ser Gly Ile Leu Ser   60
2270 TTG AAT GAG GAA GGG AAA GCT TCA CAA CAT GTT CAG GCA CTA CCT TCA GGT ATT CTG TCA

       Leu Asp Cys Ala Ile Gly Ile Gly Gly Tyr Pro Lys Gly Arg Leu Ile Glu Leu Phe Gly   80
2330 TTA GAT TGC GCA ATT GGT ATC GGT GGT TAT CCT AAA GGA CGA CTC ATT GAG CTA TTT GGA

       Ala Glu Ser Ser Gly Lys Thr Thr Val Ala Leu Gln Ala Val Ala Glu Thr Gln Lys Asn   100
2390 GCA GAA TCC TCT GGA AAA ACA ACT GTA GCG CTC CAA GCA GTA GCT GAA ACA CAA AAA AAC

       Gly Gly Tyr Val Ala Tyr Ile Asp Ala Glu Asn Ser Leu Asp Ile Glu Tyr Ala Glu Asn   120
2450 GGG GGT TAT GTG GCT TAT ATT GAT GCT GAA AAC TCA CTT GAT ATA GAA TAT GCT GAA AAC

       Leu Gly Val Lys Ser Asp Ser Leu Ile Phe Ala Gln Pro Asp Thr Gly Glu Glu Ala Phe   140
2510 CTC GGT GTC AAA TCA GAT AGT CTA ATA TTT GCT CAA CCT GAT ACA GGA GAA GAG GCT TTC

       Tyr Met Ile Asn Glu Phe Val Arg Thr Gly Ala Phe Asp Leu Ile Val Val Asp Ser Val   160
2570 TAT ATG ATT AAT GAA TTT GTT AGA ACA GGA GCT TTT GAC TTA ATT GTA GTG GAT TCA GTT

       Ala Ala Leu Thr Pro Ala Ser Glu Ile Asp Gly Val Lys Met Pro Gly Gln Gln Ala Lys   180
2630 GCA GCA CTC ACT CCT GCT TCT GAG ATA GAT GGT GTT AAA ATG CCT GGT CAA CAA GCT AAA

       Met Met Ser Glu Gln Leu Ser Gln Leu Val Gly Lys Val Asn Gln Thr Lys Thr Val Ile   200
2690 ATG ATG TCT GAG CAA CTT TCA CAG TTA GTA GGT AAA GTT AAC CAA ACA AAG ACA GTA ATC

       Ile Phe Ile Asn Gln Ile Arg Ser Thr Met Ser Gly Leu Phe Leu Asn Lys Glu Thr Thr   220
2750 ATC TTC ATC AAC CAA ATA CGC TCT ACC ATG AGT GGC TTG TTC TTA AAT AAA GAA ACC ACC

       Pro Gly Gly Ser Ala Leu Lys Phe Tyr Ser Ser Val Arg Ile Glu Val Lys Ser Gly Glu   240
2810 CCA GGT GGT TCG GCT CTA AAA TTC TAT TCA TCT GTT CGT ATC GAA GTG AAA TCT GGA GAA

       Lys Ile Lys Asp Gly Ile Asp Thr Ile Gly Lys Lys Thr Thr Leu His Thr Val Lys Asn   260
2870 AAA ATT AAA GAT GGA ATA GAT ACC ATT GGT AAA AAA ACA ACA CTT CAT ACA GTC AAA AAT

       Lys Val Ser Ser Pro Tyr Lys Lys Pro Thr Val Ile Asn Ile Phe Gly Asp Gly Phe Ser   280
2930 AAG GTT TCA TCG CCC TAT AAA AAG CCA ACT GTT ATT AAT ATT TTT GGA GAC GGA TTT TCT

       Gln Glu Ile Asp Val Val Thr Thr Ala Leu Gln Leu Gly Val Val Lys Lys Leu Gly Glu   300
2990 CAA GAA ATT GAT GTC GTT ACA ACT GCA CTT CAA CTT GGA GTA GTA AAG AAG CTA GGC GAA

       Trp Tyr Ser Phe Asn Gly Gln Lys Leu Gly Arg Gly Ile Phe Gly Val Lys Glu Tyr Leu   320
3050 TGG TAT TCA TTT AAT GGA CAA AAA TTA GGA CGT GGA ATT TTC GGA GTA AAA GAA TAT CTC

       Ser His His Pro Ser Val Phe Asn Ala Leu Asp Asn Leu Thr Arg Glu Ala Leu Gln Phe   340
3110 TCT CAT CAT CCT TCG GTT TTT AAT GCA CTA GAT AAC TTA ACT CGT GAA GCT TTG CAA TTT

       Ser Stop   341
3170 TCA TAA TAAAAGGGGACTAAATTTAGCCCCCTTTTTTCCCATCTCAATTAACTTAACGAAAAAGAATAGTATTCTTG
                ━━━━━━━━━━━━━━━━━━━▶◀
                ΔG=-25 2 kcal/mole
3247 AAACTTGTTGATTTTATAGGAATTGAAGGATACAAGTTCGTCAATTATGATATAAAAATAACAACTTAAAAATATTGTT

3326 CTTATAATCTTTA

kcal/mole, 1 bp downstream of the ochre stop codon, has the potential to form a hairpin loop structure and thus could serve as a transcriptional terminator The overall GC content is 36% which is comparable to the 37% average for lactococcal genes This plasmid-encoded recA was designated $recA_{LP}$ (for lactococcal plasmid) to distinguish it from the chromosomally-located lactococcal recA ($recA_L$) identified by Duwat et al (1992)

The second, larger ORF, designated ORFU (Fig 6 1 and Fig 6 2, first ORF), is located 400 bp upstream of the ATG start codon of $recA_{LP}$ It is 1464 bp in length and has the capacity to encode a protein with a predicted molecular mass of 55 9 kDa A putative RBS (GGAGG) with a ΔG value of -14 4 kcal/mole was found 5 nt upstream of the ATG start codon A consensus -10 sequence (TATAAT) was identified 37 bp upstream of the RBS separated by 17 bp from a -35 sequence (TTGATT) The dinucleotide TG identified in many lactococcal promoters immediately precedes the -10 sequence No obvious transcriptional terminator was identified after the opal stop codon The overall GC content for this ORF is 35% which is marginally lower than the 37% average for lactococcal genes Comparison of the DNA sequence of ORFU with known sequences in the EMBL and Genbank databanks (release 82) using the DNAstar software programme (DNAstar INC , Madison, WI) revealed no significant DNA homology

As expression of recA is regulated by LexA in *E coli*, a search of the promoter regions of recA and ORFU for sequences resembling the consensus *E coli* LexA binding site (t a C T G T a t a t a n a n a C A G t a) was performed Three sequences, which partially agree with this motif, are located within the promoter of ORFU (Fig 6 2)

6.2.2 Amino acid sequence analysis.

Comparison of the deduced amino acid sequence of $RecA_{LP}$ with the RecA's of

E coli and four gram positive bacteria demonstrated 40-46% identity and up to 89% conservation overall The RecA's of *Streptococcus pneumoniae* and *B subtilis* showed the highest level of identity and conservation, respectively with RecA$_{LP}$ Homology was as low as 18% at the termini increasing to 60% in the protein core In contrast to the complete amino acid sequence identity shared by the three lactococcal RecA's identified by Duwat *et al* (1992), RecA$_{LP}$ showed only 45% identity and 86% conservation with these proteins Figure 6 3 illustrates the amino acid sequence comparison of RecA$_{LP}$ and five other RecA proteins

In recent years, the RecA proteins of more than fifty bacteria, both gram positive and gram negative, have been analysed at the DNA sequence level Comparison of their deduced amino acid sequences has revealed regions which are highly conserved and thus, are believed to be functionally important All sequences to date have contained a 9-amino acid RecA signature motif A nonapeptide (A-L-K-F-Y-S-S-V-R) which conforms to this consensus sequence (A-L-K-F-F/Y-S/T/A-S/T/A-V-R) is located from aa 225 to aa 233 on RecA$_{LP}$ (Fig 6 3) In addition, a P-loop motif (G-A-E-S-S-G-K-T) conforming to the consensus ATP-binding motif (G/A-x-x-x-x-G-K-T/S) found in all RecA's is located from aa 80 to aa 87 (Fig 6 3)

Studies by Story *et al* (1992) on the RecA protein of *E coli* have suggested a model for its structure *in vivo* which identifies two motifs or loops (L1 and L2) believed to be responsible for double-stranded and single-stranded DNA binding, respectively L1 extends from aa 157-164 and L2 from aa 195-209 in the *E coli* RecA These loops, however, are not well conserved in RecA$_{LP}$ unlike RecA$_L$ In fact the region L1 is three amino acids shorter in the plasmid-encoded version (Fig 6 3)

Comparison of the deduced amino acid sequence of ORFU with known sequences in the Swiss and PIR and translated protein databases using the DNAstar software programme (DNAstar INC , Madison, WI) showed no significant homology No peptide motifs were identified from the Prosite database (release 11) using the

```
1   M E Q P Q Y N S Y K - - V R K L D D P E E K K L A I L K A T Q S T E K F G S N T I L N E E G K A S Q H V Q A L P S G I   RecA(LP)
1   M A T K K K T N F D D I T K K Y G A E R D K A L A D - - A L A L I L R D I G K G S L M R L G E A A N Q K V S V V S S G S   RecAL
1   M - - - - - - - - - - - - - - - S D R Q A A L D M - - A L K Q I E K Q F G K G S I M K L G E K T D T R I S T V P S G S   RecAB
1   M - - - - - - - - - - - - - - - D N D R Q K A L D T - - V I K N M F S F G K G A V K L G D N I G R R V S I T S T G S   RecASA
1   M A - K K P K K L E E I S K K F G A E R E K A L N Q - - A I K L T F K D F G K G S I M R L G E R A E Q K V Q V M S S G S   RecSP
1   A - - - - - - - - - - - - - - - I D E N K Q K A L A A - - A L G Q I E K Q F G K G S I M R L G E D R S M D V E T I S T G S   RecAEc

59  L S E D C A I G I G G Y P K G R L I E L F G A E S S G K T T V A L Q A V A E T Q K N G G Y V A Y I D A E N S L D I E Y A   RecA(LP)
59  L A L D I A L G A G G Y P K G R I V E I Y G P E S S G K T T V A L H A V A A V Q K E G G I A A F I D A E N A L D P E Y A   RecAL
43  T A L D T A I G I G G Y P R G R I I E V Y G P E S S G K T T V A L H A I A E V Q Q R T - S A F I D A E H A L D P V Y A   RecAB
44  V T L D N A I G V S G G Y P K G R I I E I Y G P E S S G K T T V A L H A I A E V Q S N G G V A A E I D A E H A L D P I Y A   RecASA
58  L A L D I A L G S S G G Y P K G R I I E I Y G P E S S G K T T V A L H A V A Q A Q K E G G I A A F I D A E H A L D P A Y A   RecSP
45  I S L D I A L G A G G L P M G R I V E I Y G P E S S G K T I L T L Q V I A A A Q R E G K T C A F I D A E H A L D P I Y A   RecAEc
```
<div align="center">P-loop motif</div>

```
119 E N E G V K S D S I F A Q P D I G E E A F Y M I N E F V R T G A F D I I V V D S V A A L T P A S E I D G V - - - K M P   RecA(LP)
119 K A L G V N I D E L L I S Q P D T G E Q G I Q I A E K L I T S G A V D L V I V D S V A A L V P K A E I D N E I G D S S V   RecAL
102 Q K L G V N I E E L L L S Q P D T G E Q A L E I A E A L V R S G A V D I I V V D S V A A L V P K A E I E G D M G D S H V   RecAB
104 A L G V D I D N L Y L S Q P D H G E Q G L E I A E A F V R R G A V D I I V V D S V A A L T P K A E I E G E M G D S H V   RecASA
118 A A L G V N I D E L L L S Q P D S G E Q G L I I A G K I D S G A V D I V V V D S V A A L V P R A E I D G D I G D S H V   RecSP
105 R K L G V D I D N L L C S Q P D T G E Q A L E I C D A L A R S G A V D V I V V D S V A A L T P K A E I E G E I G D S H M   RecAEc
```
<div align="right">loop L1</div>

```
176 G Q Q A K M M S E Q L S Q L V G K V N Q T K V I T F I N Q I R S T M S G L F L N K E T T P G G S A L K F Y S S V R I E   RecA(LP)
179 G L Q A R M M S Q A M R K L A G H I N K T K T T A I F I N Q L R E K V G V M I G S P E T T P G G R A L K F Y A S V R I D   RecAL
162 G L Q A R L M S Q A L R K L S G A I N K S K T I A I L I N Q I R E K V G V M F G N P E T I P G G R A I K F Y S S V R I E   RecAB
164 G L L A R L L M S Q A L R K L S G A I S K S N I T A I F I N Q I R E K V G V M F G N P E I I P G G H A L K F Y S S V R I E   RecASA
178 G L Q A R M M S Q A M R K L G A S I N K T K I I A I F I N Q L R E K V G V M F G N P E T T P G G R A L K F Y A S V R I D   RecSP
165 G L A A R M M S Q A M R K L A G N L K Q S N I L L I F I N Q I R M K I G V M F G N P E T T T G G N A L K I Y A S V R I D   RecAEc
```
<div align="center">loop L2   RecA signature motif</div>

236 V K S G E K I K D G I D T - - - - I G K K T T L H T V K N K V S S P Y K K P T V I N I F G D G F S Q E I D V V T T A L Q RecA(LP)
239 V R G S T K I E E G S G D N K T Q I G K I T K I K V V K N K V A P P F K V A L V D I M F G E G I S S T G E L L N I A V E RecAL
222 V R R A E Q L K Q G N D - - - - V M G N K T K I K V V K N K V A P P F R T A E V D I M Y G E G I S K E G E I I D L G T E RecAB
224 V R R A E Q L K Q G Q E - - - - I V G N R T K I K V V K N K V A P P F R V A E V D I M Y G Q G I S K E G E L I D L G V E RecASA
238 V R G N T Q I K G T G D Q K E T N V G K E T K I K V V K N K V A P P F K E A V V E I M Y G E G I S K T G E L L K I A S D RecSP
225 I R R I G A V K E G E N - - - - V V G S E I R V K V V K N K I A A P F K Q A E F Q I L Y G E G I N F Y G E L V D L G V K RecAEc

292 L G V V K K L G E W Y S F N G Q K L G R G I F G V K E Y L S H H P S V F N A L D N L T R E A L Q F - - - - - - - - - - - - RecA(LP)
299 E G I I K K S G A W F A Y N D E K I G Q G A E K A K N Y L K E H Q D V F D E I D H K V R A A H G L L L D D S E V A E T E E RecAL
278 L D I V Q K S G S S W Y S Y E E E R L G Q G R E N A K Q F L K E N K D I M L M I Q E Q I R F H Y G L D N N G V V Q Q Q A E RecAB
280 N D I V D K S G A W Y S Y N G R M G Q Q G K E N V K M Y L K E N P Q I K E F I D R K L R E K L G I S D G D V - - E E T E RecASA
298 L D I I K K A G A W Y S Y K D E K I G Q G S E N A K K Y L A E H P E I F D E I D K Q V S K F G L I D G E E V S E Q D T RecSP
281 E K L I E K A G A W Y S Y K G E K I G Q G K A N A T A W L K D N P E T A K E I E K K V R E L L - L S N P N S T P D F S V RecAEc

341 - S RecA(LP)
359 E T T - A F K N RecAL
338 E T Q E E - L E F E E RecAB
338 D A P K S - L F D E E RecASA
358 E N K K D E P K K E E A V N E E V P L D L G D E L E I E I E E RecSP
340 D D S F G V A E T N E D F RecAEc

Boxed areas indicated complete aa sequence identity. Regions involved in ATP-binding (P-loop), double-stranded DNA-binding (loop L1) and single-stranded DNA-binding (loop L2), and the RecA signature motif are indicated.

MacPattern programme giving no insight into the function of ORFU Analysis of the hydrophobicity of this protein using the method of Kyte and Doolittle showed no regions of marked hydophobicity leading us to propose a cytosolic location

Codon usage analysis is being used increasingly as an indicator of gene expression levels A survey of 100 genes has indicated the optimal codon usage pattern in *Lactococcus* The frequency of optimal codon usage (FOP) for $recA_{LP}$ is 0 36 as compared to 0 65 for $recA_L$ (John Peden, personal communication) which suggests a moderate level of expression for $recA_{LP}$, with $recA_L$ expressed at a comparatively high level However, it must be remembered that $recA_{LP}$ is present in multiple copies in a cell relative to a chromosomally-encoded *recA* gene These FOP values are based on a scale of 0 0 to 1 0 with a ribosomal protein, which would be expected to have a high level of expression, having the highest value (0 85) yet observed in *Lactococcus* The FOP for ORFU is 0 33 which also suggests a moderate level of expression for this gene

6.2.3 Complementation Studies

VEL1122 is a RecA deficient derivative of *L lactis* ssp *lactis* MG1363 generated by Duwat and Gruss (1994) by replacement recombination To assess if $RecA_{LP}$ had the ability to complement the RecA mutation, a cloned fragment of pNP40 encoding the $recA_{LP}$ gene was introduced into VEL1122, generating the strain PG030 Activity of RecA can be assessed in a number of ways The co-protease function, for example, can be investigated by inducing DNA damage by exposure to mitomycin C Both VEL1122 and PG030 failed to produce colonies on GM17 containing 50 ng/ml mitomycin C whereas MG1363 grew as normal

6.2.4 Does RecA play a role in bacteriophage resistance?

The proximity of $recA_{LP}$ to the phage resistance determinants on pNP40, and

the involvement of RecA in abortive infection in other bacteria, prompted an assessment of the phenotypic expression of AbiE and AbiF in the RecA deficient host Following introduction of the phage resistance genes into VEL1122, the efficiencies of plaquing of phages øc2 and ø712 were evaluated The results presented in Table 6 1 show that while expression of AbiE is independent of RecA, the resistance conferred by AbiF in VEL1122 is reduced relative to that conferred in MG1363 Interestingly, the pNP40-encoded *recA* gene was unable to complement the function performed by RecA$_L$ in phage resistance

6.2.5 Distribution of *recA*$_{LP}$ in lactococci

As the plasmid-location of *recA*$_{LP}$ was unusual, and as the amino acid sequence of RecA$_{LP}$ was comparatively dissimilar to that of RecA$_L$, *L lactis* ssp *lactis* biovar *diacetylactis* DRC3 (the parent strain harbouring pNP40) and 9 other wild-type lactococcal strains were investigated using PCR for the presence of *recA*$_{LP}$-like and *recA*$_L$-like sequences In addition to DRC3, two other strains (UC317 and UC503) gave rise to PCR products of equivalent size (Table 6 2) Interestingly, no PCR products were obtained for these three strains using primers complementary to the chromosomally-encoded *recA* gene of Duwat *et al* (1992) although PCR products corresponding to the *recA*$_L$ gene were obtained for ML3 (the strain from which *recA*$_L$ was originally sequenced), three other strains shown by Lucey *et al* (1992) to be closely related to ML3 (712, 952 and C2) and also for UC653 (Table 6 2) It is possible, however, that the absence of *recA*$_L$-like PCR products for DRC3, UC317 and UC503, is due to primer mismatching, reflecting minor differences in the DNA sequence of *recA*$_L$ for these strains and not the absence of a second *recA* gene

6 3   DISCUSSION

Table 6.1

EOP and plaque sizes of phages øc2 and ø712 on Rec+ and Rec- *L lactis* ssp. *lactis* MG1363 strains containing AbiE and AbiF

Resistance	Rec+		Rec-	
	EOP	Plaque size	EOP	Plaque size
øc2				
none	1 0	2-3mm	1 0	2-3mm
AbiE	1 0	2-3mm	1 0	2-3mm
AbiF	3 0x10^{-4}	pinpoint-1mm	1 1x10^{-1}	pinpoint-1mm
AbiF+RecA(LP)+ORFU	3 0x10^{-4}	pinpoint-1mm	1 1x10^{-1}	pinpoint-1mm
pNP40	NP	-	ND	-
ø712				
none	1 0	1mm	1 0	1mm
AbiE	3 0x10^{-4}	0 5-1mm	7 0x10^{-4}	0 5-1mm
AbiF	2 3x10^{-6}	pinpoint	<1 0x10^{-5}	-
pNP40	NP	-	ND	-

NP-no plaques; ND-not done; Rec+ host=MG1363; Rec- host=VEL1122, a derivative of MG1363 generated by Duwat and Gruss (1994)

Table 6.2

Presence of *recA_L* and *recA_{LP}* in *Lactococcus lactis* stains

Strain	RecA(L)	RecA(LP)
MG1614	+	-
MG1614/pNP40	+	+
DRC3	-	+
18-16	-	-
UC317	-	+
UC503	-	+
UC563	+	-
HO2	-	-
C2	+	-
712	+	-
ML3	+	-
952	+	-

This paper reports the cloning and DNA sequence analysis of a plasmid-encoded *recA* homologue (designated *recA*$_{LP}$) from *Lactococcus* It compares the deduced amino acid sequence and functionality of this gene with the chromosomally-encoded lactococcal *recA* gene (*recA*$_L$)

To our knowledge, this is the first *recA* homologue located to a plasmid, however, the determinants for many important functions in lactococci are located on plasmids with extra-chromosomal elements accounting for up to 10% of the total cellular DNA In this study, two additional lactococcal strains were found to contain *recA*$_{LP}$-like sequences although a plasmid-location has yet to be confirmed for these Interestingly, when tested using *recA*$_L$-specific primers, DRC3 and both of these strains failed to yield PCR products corresponding to *recA*$_L$ although Duwat *et al* (1992) had shown a high level of conservation among lactococcal *recA* genes

In complementation studies, RecA$_{LP}$ failed to complement the RecA deficiency in *L lactis* ssp *lactis* VEL1122, as assessed by resistance to mitomycin C It is possible, however, that functions of RecA, other than co-protease activity, are provided by this protein as the lactococcal RecA was shown by Duwat and Gruss (1994) to have a role in the management of other forms of stress in the cell

In *V cholerae* and *E coli*, there have been suggestions of RecA involvement in phage abortive infection mechanisms In *V cholerae* biotype El Tor (Biswas *et al*, 1992), RecA is directly implicated where it cleaves the *sulA* gene product *sulA* is one of two genes which together are responsible for the abortive response to phage infection of El Tor hosts There has also been a suggestion that LexA may be involved in regulation of an *abi* gene encoded by the *E coli* plasmid ColIb (Gupta and McCorquodale, 1988) In this study, it was found that the chromosomally-encoded RecA was essential for full phenotypic expression of AbiF This was not as a result of direct proteolytic cleavage of AbiF as it does not contain the Ala-Gly bond necessary

100

for protease activity of RecA As three putative LexA binding sites were identified in the promoter region of ORFU, which is divergently transcribed relative to $abiF$ on pNP40, it is possible that LexA is also involved in the regulation of this gene During phage infection, phage-induced degradation of host DNA could result in induction of the host-encoded $recA$ - In the absence of RecA, however, it would be expected that cleavage of the LexA repressor would not take place and that the $abiF$ gene would not be expressed Interestingly, while $RecA_L$ has a role in phage resistance, $RecA_{LP}$ does not supply this function when introduced into VEL1122

It is also interesting to note that, while pNP40 confers complete resistance to all phage tested in MG1363, in its native strain DRC3 a phage ødrc3 is capable of propagating efficiently The absence of a $recA_L$-like sequence in this strain may explain why AbiF is not as effective there Another notable feature of pNP40 is that in MG1363 it confers only partial resistance to phage at 37°C As RecA in $Lactococcus$ plays a role in thermal stress, it is possible that there is a link between the temperature sensitivity of the pNP40-encoded phage resistance and RecA

Finally, in S $pneumoniae$ and in lactococci, $recA$ forms part of a polycistronic operon (Martin et al , 1994, Duwat and Gruss, 1994) In lactococci, two $recA$ transcripts were observed, one of which includes the upstream formamidopyrimidine-DNA-glycosylase (fpg) gene and in S $pneumoniae$, a second ORF, $exp10$, which encodes a membrane-located protein is co-transcribed The absence of a transcriptional terminator following ORFU on pNP40 may indicate transcriptional readthrough to the terminator after $recA_{LP}$ Thus, ORFU and $recA_{LP}$ could potentially be co-transcribed Analysis of the mRNA content of hosts containing ORFU and $recA_{LP}$ could establish if this actually occurs

CHAPTER VII

GENERAL DISCUSSION

Bacteriophage interference with the starter culture(s) used in dairy fermentations can cause slow acid production which can result in an inferior product or, in more extreme cases, complete starter failure. The introduction of selected phage resistance plasmids by conjugation into commercial starter strains is one approach that has proven to be successful in minimising disruption by phage. It is of interest both from a fundamental and commercial perpective to establish the underlying mechanisms which contribute to the phage insensitivity encoded by these plasmids. This, in turn, will allow for the development of knowledge-based strategies for the combination of multiple defences in starter strains. Furthermore, molecular characterisation of resistance genes is a prerequisite to their introduction into commercial strains using food-grade cloning protocols.

This study was undertaken to investigate the phage resistance mechanisms encoded by the lactococcal plasmid pNP40, a 65 kb plasmid originally identified by McKay and Baldwin (1984) in *L. lactis* ssp *lactis* biovar. *diacetylactis* DRC3. This conjugative plasmid has been exploited to improve the phage resistance properties of *L. lactis* ssp. *lactis* biovar. *diacetylactis* DPC220, a strain used in lactic butter fermentation (Harrington and Hill, 1991). Introduction of pNP40 into the plasmid-free strain *L. lactis* ssp. *lactis* MG1614 conferred an impressive level of resistance to phage, with no plaques obtained on a lawn of cells using either prolate- or small isometric-headed types (McKay and Baldwin, 1984; Chapter 3).

Prior to this study, numerous examples of each of the three categories of phage resistance identified in lactococci have been described. In the case of abortive infection, only one gene was characterised at the DNA sequence level although subsequently, two additional *abi* genes have been reported. In this thesis, we report the characterisation of the determinants for two additional Abi mechanisms which are encoded by pNP40 and furthermore, a third novel mechanism, penetration blocking, is also described.

In Chapter 3, an abortive infection system (AbiE) active against the small isometric-headed phage ø712 was identified. This had little effect on phage DNA

replication suggesting that it operates late in the lytic cycle, possibly during transcription/translation, phage packaging or release In the case of AbiC, an abortive mechanism which was also found to act late in the lytic cycle (Durmaz *et al* , 1992), it was established, using antibodies directed against a phage capsid protein, that production of phage structural proteins was inhibited (Moineau *et al* , 1992) A similar approach, based on the detection of phage-specific proteins, or on the detection of phage-specific mRNA would further establish the basis of the AbiE mechanism An alternative approach could exploit a ⌀712 mutant which was found to be capable of plaquing efficiently on hosts containing AbiE (Chapter 3) In other systems, when mutant phage were examined, it was shown that they had acquired additional DNA which rendered them insensitive to the phage resistance mechanism In the case of phage resistance to pTR2030-containing transconjugants, this additional DNA originated from the *Lla*I methylase gene of pTR2030 (Hill *et al* , 1991b) while for an AbiC-resistant mutant phage isolated by Moineau *et al* (1994), it was shown that recombination with specific chromosomal DNA sequences was responsible Molecular characterisation of the mutant ⌀712 would establish whether a point mutation or a recombination event was responsible for insensitivity to AbiE Analysis of the AbiE-resistant derivative of ⌀712 may also indicate the target of inhibition for this mechanism on the phage genome

DNA sequence analysis of abortive infection determinants has, in general, provided little information regarding the the nature of the resistance This is also the case for *abiE,* however, one unusual feature of this determinant is that it appears that two ORF's are required for expression of resistance This is the first description of an Abi mechanism which required two ORF's for activity It remains to be established if these two putative proteins function as a heterodimer *in vivo* or act in concert in their mediation of phage resistance The availability of data for a number of *abi* genes has, however, indicated that all display GC contents atypical for lactococci and *abiEi* and *abiEii* conform to this trend. Horizontal gene transfer from other organisms has

previously been suggested when DNA of atypical GC content was identified but it is still unclear if there is a link between the low GC content and phage resistance

In Chapter 4, a second abortive infection system (AbiF) was identified on pNP40 which has a broader spectrum of activity than AbiE in that it functions against the prolate-headed phage φc2 and the small-isometric-headed phage φ712 It is likely that it acts by a general anti-phage mechanism with AbiE having a more specific target of action It also differs from AbiE in that it acts at the level of phage DNA replication In the case of AbiA from pTR2030 (Hill *et al* ,1991b), which also inhibits phage DNA replication, it was suggested that the phage origin was the target of action as it was shown that only phages harbouring a specific origin of replication were affected This is unlikely in the case of AbiF, as phages of different morphological types would be expected to have distinct *ori*'s The sequence data for *abiF*, although confirming that the gene was novel, did not contribute any insight into the mode of action of AbiF AbiF resembled the previously identified Abi systems in that a single ORF with a low GC content was responsible for the resistance phenotype

Sequence analysis of the *abi*F region identified an interesting feature downstream of the ORF A 23 bp sequence was repeated three and a half times, a motif which bears a striking organisational resemblance to the origin of replication of many theta-replicating plasmids It is possible that this region encodes a replication origin for pNP40, an hypothesis which could be confirmed by cloning of a pNP40 fragment encompassing the putative replication region into a replication probe vector Interestingly, a different plasmid origin has already been identified on pNP40 by Froseth and McKay (1991) This observation and the detection by hybridisation analysis of two copies of ISS1 on pNP40 suggests that it may be a co-integrate which could have arisen during conjugative transfer as pNP40 is self-transmissible Since the 23 bp repeated sequence is not homologous to the 22 bp repeat sequence typical of pCI305-type origins, it would, if proven to be functional, represent the first in a new family of lactococcal theta-replication origins

In Chapter 5, a completely novel phage resistance mechanism was identified which acts by preventing DNA entry into the cell, possibly by altering the phage DNA translocation activities of the cell membrane This is potentially a very powerful system since it not only allows the cell to survive despite exposure to high numbers of phage but it also titrates phage out of the environment One disadvantage of this system is that it appears to be very specific being effective, in this study, only against øc2 Assessment of the survival of other hosts containing pNP40 following exposure to their homologous phages would establish if the activity of this resistance mechanism is confined to the øc2 MG1614 phage host system Further studies on this mechanism could include analysis of the membrane proteins of hosts containing pNP40 Antibodies raised against the phage infection protein (PIP, Geller *et al* ,1993) or the 32 kDa protein which inactivates phage øc2 at the membrane level (Valyasevi *et al* , 1991) would establish if they are present in the membrane of MG1614/pNP40 Fluorescent labelling of the phage DNA and examination of infection of sensitive and resistant hosts by fluorescent microscopy would also determine whether any phage DNA enters infected resistant hosts Further subcloning of pNP40 to define the locus responsible for this resistance phenotype would permit sequence analysis of the gene(s) involved and could reveal information regarding the molecular basis of this interesting mechanism

In Chapter 6, two important discoveries are reported Firstly, a plasmid-encoded *recA* homologue (*recA*$_{LP}$) was identified on pNP40 and secondly, the chromosomally-encoded RecA protein was shown to have a role in abortive infection Although it was demonstrated that RecA$_{LP}$ played no discernible role in phage resistance and that it could not function as a co-protease in response to mitomycin C-induced DNA damage, it is possible that some of the other functions associated with RecA are retained by the plasmid-encoded homologue and that it has a role in the management of other forms of stress It seems unlikely that it performs no function in the host as it has been established that at least two other lactococcal strains carry *recA*$_{LP}$-

like sequences

The role of RecA in phenotypic expression of AbiF is particularly intriguing In Chapter 6, it was proposed that RecA involvement was at the level of gene expression It was suggested that putative LexA binding sites present upstream of the *abiF* promoter could be involved in regulation of this gene and in response to phage-induced DNA damage, RecA cleavage of the LexA repressor could result in de-repression of *abiF* Transcriptional analysis of *abiF* in a RecA deficient host would establish if this protein functions at this level Moreover, it would be interesting not only to examine transcription of *abiF* at 37°C in MG1614 or in its parent strain DRC3 but also to examine the resistance encoded by pNP40 in the RecA deficient host VEL1122 An alternative possibility is that RecA functions at the level of the mechanism of resistance

In the past five years, there has been a dramatic shift in the approach used to study phage host interactions which can be attributed, in large measure, to the advances in the application of molecular technology to lactococci Several phage resistance determinants (encoding both R/M and Abi) and, more recently, complete phage genomes have been analysed at the DNA sequence level The availability of such a large amount of data is bringing us closer to an understanding of the molecular interactions between phage and host It is anticipated that this information will contribute to the formulation of rational strategies for the construction of improved starter strains for the dairy industry

The current approach employed in lactococcal strain improvement involves the introduction of self-transmissible phage resistance plasmids into starter strains by food-grade conjugal transfer The improved understanding of the molecular basis of phage resistance mechanisms has already permitted the identification of plasmids suitable for use on a rotating basis in a commercial starter culture (Sing and Klaenhammer, 1993) It is also possible to identify plasmids which encode resistance mechanisms which act at different stages in the phage lytic cycle and thus can be be expected to act in a

complementary manner when introduced in combinations into starter strains

It has been firmly established that many factors influence phenotypic expression of phage resistance systems in lactococci including gene copy number, regulatory DNA sequences, host strain and the infecting phage Unlike the situation where natural phage resistance plasmids are used to generate strains with enhanced levels of insensitivity, recombinant DNA technology has made it possible to deliberately augment the level of phage resistance encoded by a particular gene (O' Sullivan *et al*, 1993, Dinsmore *et al*, 1994) Phage DNA has also been exploited as a source of novel phage resistance mechanisms which interfere with proliferation of specific phages, e g *per* (Hill *et al*, 1990a, O' Sullivan *et al*, 1993) and antisense mRNA (Kim and Batt, 1992) and it is anticipated that the availability of sequence data for an increasing number of phage genomes will enhance the possibility of exploiting phage as a source of new phage resistance mechanisms

Another important area of current interest concerns the genetic relatedness of lactococcal phage Molecular characterisation of phage genomes has demonstrated that classification based on morphology and/or host-range was too rigid and that bacteriophage genomes are in fact quite fluid and are prone to undergo recombination events This realisation is based in part on the detection of genetically modified phages which have been isolated from industrial cheese-making operations following the prolonged use of phage resistant transconjugant starter cultures (Hill *et al*, 1991b, Moineau *et al*, 1994) In some cases, these recombinogenic phage were shown to have undergone significant structural alteration although the change in DNA content was relatively small (Moineau *et al*, 1994) This highlights the ability of phage to overcome individual phage resistance mechanisms and emphasises the inadvisability of using a single phage resistance mechanism over a prolonged period of time Rotation of resistance systems within starter strains will assist in combating this problem

Another parallel advance in lactococcal genetics has been the relatively recent development of food-grade cloning vectors These will facilitate the introduction into

commercial strains of both the novel phage resistance determinants (*per*, etc), and of the natural mechanisms whose expression has been genetically enhanced by molecular manipulation, increasing the number of approaches available for genetically improving the phage resistance properties of these strains

In many natural systems, the accumulation of multiple complementary phage resistances undoubtedly contributes to host survival Plasmid pNP40, which encodes three mechanisms acting at different stages in the lytic cycle (at phage DNA entry, DNA replication and post-DNA replication) and against different phages, is an example of how multiple complementary systems have been stacked The location of this combination of phage resistance systems on a conjugative plasmid makes pNP40 an ideal candidate for use in the improvement of starter cultures Alternatively, the sequence data now available for the genes encoding AbiE and AbiF permits their introduction into commercial strains by recombinant DNA technology using food-grade methodology This work has already been initiated employing a vector based on lactose utilisation for selection (A Coffey, personal communication) Finally, as a completely new phage resistance category, the phage DNA penetration blocking mechanism is expected to be complementary to all phage resistance systems identified to date and is therefore a useful addition to the pool of resistances available for starter strain development A further contribution of the research presented in this thesis is the identification of a role for RecA in the phenotypic expression of *abiF* which is the first description in lactococci of the involvement of a generalised host function in abortive infection

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