LACTOCOCCAL PLASMID REPLICON: VECTOR CONSTRUCTION AND GENETIC ORGANIZATION

by

FENGFENG XU

A thesis presented in partial fulfilment of the requirements

for the degree of Doctor of Philosophy in Biotechnology

at Massey University, Palmerston North

New Zealand

1990

ABSTRACT

The 5.5 kb high-copy number cryptic plasmid pDI25 from *Lactococcus lactis* subsp. *lactis* 5136 was isolated and used as the basis to construct a series of vectors. The vector pFX1 (5.5 kb) was first made by ligating the 4.5 kb *Hpa*II-*Mbo*I fragment of the lactococcal plasmid to the 1 kb chloramphenicol transacetylase gene from the staphylococcal plasmid pC194.

Plasmid pFX1 was further modified by deleting a non-essential 1.9 kb *Cla*I region to construct pFX2 (3.6 kb). Deletion analysis showed an essential region for plasmid replication was located within a 1.2 kb *Cfo*I-*Tha*I-*Cfo*I fragment.

The vector pFX3 was constructed by incorporating the α fragment of the *Escherichia coli lacZ* structural gene, a multiple cloning region and the T7 and T3 promoters from pUBS into pFX2. Recombinant plasmids constructed in *E. coli* using X-gal selection could be subsequently electroporated into lactococci. pFX3 could also be used directly for transcription studies or DNA sequencing of cloned inserts.

A set of lactococcal translational gene-fusion vectors was constructed by incorporating the *E. coli lacZ* gene fusion system (pNM480,481,482) into pFX2. These constructions, pFX4, pFX5 and pFX6, permit the fusion of cloned genes to *lacZ* in all three reading frames. Gene expression can be readily and quantitatively monitored by measuring ß-galactosidase activity.

All the pFX vectors were efficiently transformed into lactococci and *E. coli* by electroporation (10^4-10^6 cfu/μg DNA in each host) and maintained stably in both organisms (>95% cells carrying the Cm marker after 100 generations growth without drug selection).

A cell-wall bound proteinase from *Lactococcus lactis* subsp. *cremoris* H2 was isolated and characterized as a PI type proteinase since it preferentially degraded ß-casein. A 6.5 kb *Hin*dIII fragment of plasmid pDI21 (63 kb) was initially cloned and expressed this enzymatic activity in *E. coli* using vector λNM1149. The restriction map of this pDI21 *prt* gene fragment had minor differences from those of other published lactococcal *prt* fragments.

Using pFX1, the pDI21 *prt* gene fragment was recloned and directly electroporated into lactococci where it was efficiently expressed. The effectiveness of pFX3 was demonstrated by initially cloning a pDI1 4.4 kb *Eco*RI tagatose 1,6-bisphosphate aldolase gene fragment into *E. coli* from where it was electroporated into lactococci. Using the translational fusion vectors pFX4, pFX5 and pFX6, the 6.5 kb *Hin*dIII *prt* gene fragment of pDI21 was identified as having two promoters with opposite orientations. The pDI21 2.0 kb *Eco*RI galactose-6-phosphate isomerase gene fragment was shown to carry a promoter and the direction of gene transcription was determined.

The complete DNA sequence of the lactococcal portion of pFX2 (2508 bp) was determined and the genetic organization analyzed. A lactococcal plasmid plus origin and two replication protein coding regions (*repA* and *repB*) were located. RepA had an αhelix-turn-αhelix motif, a geometry typical of DNA-binding proteins. RepB showed high homology to the plasmid replication initiation proteins from other Gram-positive bacteria and *Mycoplasma*. The transcribed inverted repeat sequence between *repA* and *repB* could form an attenuator to regulate pFX2 replication. Upstream of the plus origin site, and in a region nonessential for replication, a 215 bp sequence identical to the staphylococcal plasmid pE194 and carrying the RS_A site was identified. The genetic organization of this lactococcal plasmid replicon shares significant similarity with the pE194 group of plasmids.

LIST OF PUBLICATIONS

Data from this work has been published in part in the following papers.

1. Yu, P.-L., Xu, F. and Pearce, L.E. (1989). Molecular characterization of the lactose plasmid of *Streptococcus cremoris* H2. Proceedings of Eighth Australian Biotechnology Conference, p349-352. Sydney, Australia.
2. Xu, F., Pearce, L.E. and Yu, P.-L. (1989). Molecular cloning and expression of a proteinase gene from *Lactococcus lactis* subsp. *cremoris* H2 in *Escherichia coli* and *Lactococcus lactis* subsp. *lactis*. Proceedings of Annual Conference of New Zealand Microbiological Society. Hamilton, New Zealand.
3. Xu, F., Pearce, L.E. and Yu, P.-L. (1990). Molecular cloning and expression of a proteinase gene from *Lactococcus lactis* subsp. *cremoris* H2 and construction of a new lactococcal vector pFX1. Arch. Microbiol. 154:99-104.
4. Xu, F., Pearce, L.E. and Yu, P.-L. (1990). Construction of a family of lactococcal vectors for gene cloning and translational fusion. FEMS Microbiol. Lett. (in press).
5. Yu, P.-L., Pearce, L.E. and Xu, F. (1989). Improved recombinant DNA method. New Zealand Patent 229125.
6. Yu, P.-L., Xu, F. and Pearce, L.E. (1990). Gene cloning and vector constructions in fermentative lactococci. p15-21. In: Fermentation Technologies. Industrial Applications. Yu, P.-L. (ed.), Elsevier Applied Science, UK.
7. Xu, F., Pearce, L.E. and Yu, P-L. (1990). Lactococcal replicon: vector constructions and genetic organization. Proceedings of Annual Conference of New Zealand Microbiological Society. Lincoln, New Zealand.
8. Xu, F., Pearce, L.E. and Yu, P.-L. (1990). Sequence analysis of a lactococcal plasmid replicon. Mol. Gen. Genet. (submitted).
9. Xu, F., Yu, P.-L. and Pearce, L. E. (1990). Lactococcal plasmid replicon: vector construction and genetic organization. In: Streptococcal Genetics. American Society for Microbiology, Washington, DC, USA (in press).

ACKNOWLEDGEMENTS

I wish to thank my chief supervisor Dr. Pak-Lam Yu and associate supervisor Dr. Lindsay Pearce for their constant encouragement, advice and guidance throughout this thesis.

I gratefully acknowledge New Zealand Dairy Research Institute (NZDRI) and the Department of Biotechnology for providing the facilities which enabled this study. This work was supported by a NZDRI-Massey University research contract to Dr. Pak-Lam Yu.

I am indebted to Drs. Audrey Jarvis, Graham Davey, Chris Pillidge and Lawrence Ward for their great interest and many helpful discussion during the course of this study.

I would like to thank many present and past colleagues, particularly Charlotte, Samantha, Petrena and Anna at NZDRI, and Kerrie, Sirinda, Derek, Chanxing and technicians in the Department of Biotechnology for their friendship and technical asistance.

My thanks extend to the Applied Biochemistry and Protein Chemistry sections at NZDRI for the assay of ß-galactosidase and the supply of purified casein fractions respectively.

I would also like to thank Dan Legg for tutoring in the use of the computer facilities, Paul Le Ceve for his expertise with the photo reproductions and Chris Thomasen for her excellent skill in preparing the illustrations.

Finally, I wish to thank my family and in-laws, and particularly my wife for their constant concern, love and support.

LIST OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

ABBREVIATIONS

kb	kilobases
bp	basepairs
pfu	plaque forming units
cfu	colony forming units
Ap	ampicillin
Cm	chloramphenicol
SDS	sodium dodecyl sulphate
MW	molecular weight
kDa	kilodaltons
PEG	polyethylene glycol
X-gal	5-bromo-4-chloro-3-indoyl galactopyranoside
IPTG	Isopropyl thiogalactopyranoside
ORF	open reading frame
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]