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MOLECULAR ANALYSIS
OF
THE LACTOSE METABOLISING GENES
FROM
LACTOCOCCUS LACTIS

A thesis presented in partial fulfilment of the requirement for the degree of PhD in Biotechnology at Massey University New Zealand.

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ABSTRACT

Two lactococcal strains possessing different lactose metabolism systems were chosen for the molecular analysis of lactose metabolising genes from *Lactococcus lactis*. These were *Lactococcus lactis* ssp. *cremoris* strain H2 and *Lactococcus lactis* ssp. *lactis* strain ATCC 7962. An attempt was made to sequence a previously cloned 4.4 kb *Eco*RI fragment of pDI21 reported to encode D-tagatose 1,6-bisphosphate aldolase. A comparison of sequence data generated from this fragment with DNA sequences in the GenEMBL data base revealed that the clones provided for this study were not lactococcal DNA but were chromosomal DNA of *E. coli* which encoded genes involved in purine biosynthesis. This part of the research programme was therefore abandoned.

The aim of the second part of this study programme was to clone and characterize the ß-galactosidase gene from plasmid pDI3, an uncharacterized plasmid of *Lactococcus lactis* ssp. *lactis* strain ATCC 7962. This was of interest as most lactococcal bacterial strains metabolize lactose by way of the Lac-PEP:PTS system and possess high phospho-ß-galactosidase activity, whereas strain 7962 metabolizes lactose by way of the lactose permease system and possesses high ß-galactosidase activity and low phospho-ß-galactosidase activity. Previous plasmid curing experiments indicated that the ß-galactosidase activity of strain 7962 is associated with pDI3. DNA hybridization work between pDI3 and a previously cloned DNA fragment containing the ß-galactosidase gene of another Gram-positive genus, *Clostridium acetobutylicum* (*cbgA*) showed that pDI3 contains DNA sequence that is to some extent homologous to ß-galactosidase sequence.

Initial experiments were carried out to confirm the involvement of pDI3 on strain 7962's ß-galactosidase activity. Strain ATCC 7962 wildtype containing four plasmids and the strain cured of three other plasmids (i.e. derivative strain of 7962 containing only pDI3) exhibited a Lac$^+$ phenotype, while the strain cured of all four plasmids exhibited a Lac$^-$ phenotype.

As part of the mapping strategy and the attempt to clone the ß-galactosidase gene from strain 7962, various fragments of pDI3 were cloned. Hybridization experiments using the cloned pDI3 fragments as DNA probes were also carried out to confirm the arrangement of pDI3 fragments. A physical map of pDI3 was constructed using the restriction enzymes *Bam*HI, *Pst*I, and *Sal*I. The size of pDI3 was confirmed to be 70 kb and it may contain some small repeat sequences. Some *Eco*RI restriction sites contained in pDI3 were also determined.

Several approaches were used to localize and identify the ß-galactosidase gene on pDI3. Southern hybridizations were first carried out using the *cbgA* gene. The *cbgA* gene showed weak homology to a 4.3 kb *Eco*RI doublet from pDI3. Two redundant oligonucleotide probes were designed from the highly conserved domain of deduced amino acid sequences of the available ß-galactosidase sequences from other closely related Gram-positive bacteria as well as some deduced amino acid sequences derived from ß-galactosidase sequences of Gram-negative bacteria. The 4.3 kb *Eco*RI doublet and a 4.3 kb *Hin*dIII doublet exhibited weak homology to these probes.

On the basis of these results one of the 4.3 kb *Eco*RI fragments was subsequently cloned and transformed into a Lac^- *E. coli* strain, MC1022. The cloned 4.3 kb *Eco*RI fragment (the 4.3a fragment) was shown to cross hybridize to one of the oligonucleotide probes, but did not show ß-galactosidase activity. The 4.3a kb *Eco*RI fragment was also cloned into pBR322 and transformed into another Lac^- *E. coli* host (JM109) and no ß-galactosidase activity was detected.

Based on the pDI3 physical map constructed, the 4.3a kb *Eco*RI fragment was shown to overlap a 13.6 kb *Sal*I fragment. This fragment was cloned into pBR322 and the plasmid designated pSY303, and this was transformed into *E. coli* JM109. Introduction of pSY303 into *E. coli* JM109 gave a Lac^+ phenotype. Lac^+ phenotypes were also found for other Lac^- *E. coli* host strains including *E. coli* PB2959. It was found that pSY303 expressed ß-galactosidase constitutively, however the addition of 0.1% (w/v) lactose into the medium gave a higher level of expression. An inducer for ß-galactosidase, IPTG, was not required for expression. Glucose had no repression effect on ß-galactosidase.

Some instability of the Lac^+ phenotype was observed. Transformants that were initially Lac^+ manifested phenotypic segregation into Lac^+ and Lac^- colonies. Both were found to retain the intact pSY303 plasmid and there was no difference in pSY303 DNA isolated from representative Lac^+ colonies and from representative Lac^- colonies. Some stable Lac^+ colonies were observed that became dark blue. Analysis of the plasmid from these colonies showed that pSY303 had undergone a deletion. The generation of these deletion derivatives may be a consequence of small repeat sequences.

In conclusion, a physical map of pDI3 has been constructed and the ß-galactosidase gene from pDI3 of *Lactococcus lactis* ssp. *lactis* strain ATCC 7962 was cloned and was found to express constitutively in *E. coli*, and at a higher level in the presence of lactose.

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ABBREVIATIONS

kb	kilobases
bp	basepairs
kDa	kilodaltons
MDa	megadaltons
nm	nanometres
g	gravity
rpm	revolution per minute
cfu	colony forming units
Ap	ampicillin
Cm	chloramphenicol
Em	erythromycin
Tc	Tetracycline
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
IPTG	Isopropyl thiogalactopyranoside
X-gal	5-bromo-4-chloro-3-indoyl galactopyranoside
ONPG	o-nitro-phenyl-ß-D-galactoside
ONP	o-nitro-phenyl
ORF	open reading frame
MW	molecular weight
PCR	polymerase chain reaction
PTS	phosphotransferase system
PS	permease system
Lac-PEP:PTS	lactose phosphoenolpyruvate dependent phosphotransferase system
P-ß-gal	phospho-ß-galactosidase
ß-gal	ß-galactosidase
strain 7962	a plasmid cured derivative of *Lactococcus lactis* ssp. *lactis* strain ATCC 7962 containing only one plasmid, pDI3. (is also referred to as strain NZDRI 5191
SDS	sodium dodecyl sulphate
OD	optical density
A	absorbance