Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

PEPTIDE METABOLISM IN THE LACTOCOCCI AND ITS REGULATION

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry at Massey University

IAN LINTON MOORE

1994

MOORE

Massey University Library

252
5511

Thesis Copyright Form

Title of thesis: ...Peptide Metabolism in the lactococci and its regulation...

(1) (a) I give permission for my thesis to be made available to readers in Massey University Library under conditions determined by the Librarian.

(b) I do not wish my thesis to be made available to readers without my written consent for .... months.

(2) (a) I agree that my thesis, or a copy, may be sent to another institution under conditions determined by the Librarian.

(b) I do not wish my thesis, or a copy, to be sent to another institution without my written consent for .... months.

(3) (a) I agree that my thesis may be copied for Library use.

(b) I do not wish my thesis to be copied for Library use for .... months.

Signed ....

Date ...22/12/94......

* * * * * * * * * * * * *

The copyright of this thesis belongs to the author. Readers must sign their name in the space below to show that they recognise this. They are asked to add their permanent address.

NAME and ADDRESS DATE

Abstract

Aspects of peptide metabolism in the lactococci have been investigated to increase the understanding of how these nutritionally fastidious bacteria, which have a central role in the dairy manufacturing industry, are able to grow in a complex medium such as milk.

Peptide metabolism by lactococci in milk encompasses the processes by which large oligopeptides, produced from milk-caseins by the extracellular activity of the cell wall-associated proteinase, are converted into an intracellular pool of metabolisable amino acids. This involves the activities of both membrane-bound transport systems and peptidases.

Early research into lactococcal peptide utilisation has proposed significant differences between *Lactococcus lactis* strains with respect to the mechanisms by which these bacteria utilise peptides in their environment. More recent studies of the lactococcal peptide carrier systems, based on intensive studies of only a single strain, have proposed a major role for a carrier system capable of transporting di- and tripeptides, and a subsidiary role for another system transporting oligopeptides containing four or more residues. Yet to date, peptidases with an extracellular location capable of degrading the large casein-peptides into smaller peptides have not been isolated.

This current study has attempted to investigate more fully the *in vivo* activity of the oligopeptide transport system, and to assess whether it may have a more fundamental role in peptide utilisation than previous work has suggested. For this study a model series of homologous peptides of increasing size from the dipeptide Val-Gly to the octapeptide Val-Gly_7, all based on the essential amino acid valine, was used. The larger peptides in this series, Val-Gly_3, Val-Gly_4 and Val-Gly_7, were synthesised for this work. The ability of *Lactococcus lactis* subsp. *cremoris* E8 to transport these peptides, and to grow in a chemically defined medium where they constitute the sole source of the essential amino acid valine, was studied . Preliminary peptide uptake studies were also performed using oligopeptides derived from a proteolytic cleavage of β-casein. The collective results of these studies suggest that the upper size limit, and the relative activity of this transport system, may be sufficient to permit this strain to utilise relatively large casein-derived oligopeptides without the need for hydrolysis into smaller peptides and free amino acids.

A comparative study of peptide transport by a number of *Lactococcus lactis* strains was undertaken to investigate previously published observations indicating significant differences in the mechanisms of peptide uptake between lactococcal strains.

While the results of this comparative study are consistent with the general model proposing two separate peptide carrier systems, they have revealed that significant differences can exist between strains in the relative activities and possible substrate specificities of these transport systems consistent with previous work that the lactococci have only two peptide carrier systems. These observations imply the need for caution in extrapolating the results obtained from the study of a single strain to lactococci as a whole. In contrast to the finding of significant strain differences with respect to the relative rates of peptide transport, a comparative study of the relative activity of six different intracellular peptidases showed relatively few differences in peptidase activity between strains.

An investigation was also carried out to assess whether the peptidases and transport systems involved in the utilisation of peptides were nutritionally regulated. No clear evidence was obtained for the significant induction of either the intracellular peptidase complement or the di-/tripeptide transport system.

An attempt was also made to isolate a mutant of *Lactococcus lactis* subsp. *cremoris* E8 unable to utilise dipeptides, to assess whether the di-/tripeptide transport system or the intracellular dipeptidase of this strain were essential to casein utilisation. This attempt was not successful.

Acknowledgements

The acknowledgements section of most theses is usually one of the last things to be completed, and can often be glossed over in the rush to meet submission dates. Yet in many ways this is probably one of the more important aspects of a thesis, as without the advice, encouragement and support of supervisors, colleagues, family and friends few research projects would ever reach the written stage. Therefore, while I will be taking credit for the work presented in this thesis, I would like to acknowledge the contributions of the following people.

Firstly, many thanks to the New Zealand Dairy Research Institute (NZDRI), in particular Dr Don Otter and Dr Tim Coolbear, for the generous access they gave me to the NZDRI's facilities, especially the capillary electrophoresis (CE) analytical equipment. Thanks also to Ms Carmen Norris for putting up with my clutter and the endless progression of samples that my experiments seem to generate.

I would also like to acknowledge Dr Dave Harding, Dr Darren Englebretsen, Ms Jenny Cross and Mr Grant Taylor from the Massey University Separation Science Unit, for their assistance in synthesising the peptide substrates required for this study.

Thanks are also due to Mrs Carole Flyger for performing amino acid analyses and protein sequences on samples that always seemed to be urgent. Thanks also to Mr John Allen and Mr Martin Hunt for running the FAB mass spectra.

Gratitude is also expressed to all members of my research laboratory who have helped in many ways, from acting as colleagues with which to sound ideas out with, to friends with whom I could celebrate the successes and get commiseration from for the failures. To Julian, Andrea, Mark, Edward, Cherie, Tony, John, Kathryn, Heather, Isobel, Rose, Neil, Stephen, Robyn, Treena, Jeroen, Maria, and Shaun, I will remember fondly the time we spent (trying to) pushing back the frontiers of science together. My best wishes to you all in your future research careers.

Acknowledgements are also owed to my friends outside university who were there to encourage me during the bad points and to remind me that there is a life outside academia. My thanks and love especially to Cluny, David F., David R., Bryan and Gill. A special thank you is owed to my family, especially my parents Malcolm and Helen, who provided much love and financial support during the last three years of study.

Finally, I would like to express sincere appreciation to my supervisor Associate Professor Graham Pritchard without whom the project would not have happened. Many thanks Graham for taking me on as a graduate student even though it meant an increased workload in your final years before retirement. I have learnt a lot from you, both professionally and personally over the course of this work, and hope as your last student this thesis does you justice.

Ian Moore December 1994.

List of Abbreviations

AβClA	L-alanyl-β-chloroalanine
ATP	adenosine triphosphate
β-CDM	β-casein defined medium
Boc	tertiary-butyloxycarbonyl
CDM	chemically defined medium
CHM	casein hydrolysate medium
CE	capillary electrophoresis
DIEA	di-isopropyl ethylamine
DCC	dicyclohexylcarbodimide
DCU	dicyclohexylurea
EtOH	ethanol
FITC	fluorescent isothiocyanate
Fmoc	9-fluorenylmethoxycarbonyl
HPLC	high performance liquid chromatography
SIMS	secondary ion mass spectrometry
SMM	skim milk medium
SPPS	solid phase peptide synthesis
STB	screw top bottle
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TMG	1,1,3,3-tetramethylguanidine
UBM	undefined broth medium.
Val-Gly	Valyl-glycine.
Val-Gly_2	Valyl-glycyl-glycine.
Val-Gly_3	Valyl-glycyl-glycyl-glycine.
Val-Gly_4	Valyl-$(glycyl)_3$-glycine.
Val-Gly_7	Valyl-$(glycyl)_6$-glycine.
Val-Gly_9	Valyl-$(glycyl)_8$-glycine.

Abbreviations used for amino acids:

amino acid	three letter abbreviation	one letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tyrosine	Tyr	Y
Tryptophan	Trp	W
Valine	Val	V

CONTENTS

Abstract.. ii

Acknowledgements iv

List of Abbreviations.. vi

List of Figures.. xii

List of Tables xv

Chapter 1: Introduction.. 1

1.1 The importance of lactic acid starter bacteria in the dairy industry........1

1.2 The proteolytic system of lactococcal starter bacteria........................2

1.2.1 Proteinases..2

1.2.2 Peptidases..4

1.2.3 Transport proteins..6

1.3 Peptide metabolism and its significance..11

1.4 The role of peptide metabolism in the lactococci proteolytic pathway..12

1.4.1 Current models of peptide metabolism..........................12

1.4.2 Strain differences in peptide metabolism..........................15

1.5 Aims of the current study..16

Chapter 2: Methods and Materials.. 17

2.1 Materials..17

2.1.1 Bacterial strains..17

2.1.2 Growth media constituents..17

2.1.3 Substrate for enzymes..17

2.1.4 Peptide synthesis materials..18

2.1.5 Peptide uptake materials..18

2.1.6 Miscellaneous materials..18

2.2 Methods..19

2.2.1 Growth media..19

2.2.2 Preparation of agar plates..24

2.2.3 Maintenance of bacterial cultures..24

2.2.4 Growth studies..24

2.2.5 Harvesting of cells for peptidase determinations................26

2.2.6 Peptidase assays..28
2.2.7 Protein determinations..30
2.2.8 Synthesis of Val-$(Gly)_x$ peptides................................31
2.2.9 Isolation of β-casein peptides....................................34
2.2.10 Peptide uptake experiments......................................36
(1) Using the Val-$(Gly)_x$ peptide series........................36
(2) Using the β-casein oligopeptides...........................41
2.2.11 Isolation of a lactococcal mutant defective in dipeptide utilisation...42

Chapter 3: Characterisation of peptides................................. 46
3.1 Purification and characterisation of synthetic peptides.....................46
3.1.1 Val-Gly_3...46
3.1.2 Val-Gly_4...49
3.1.3 Val-Gly_7...49
3.1.4 Val-Gly_9...53
3.2 Isolation and characterisation of β-casein peptides.........................58
3.2.1 Fractionation of the peptide products from the β-casein digestion...58
3.2.2 Confirmation of peptide purity..60

Chapter 4: Peptide utilisation by lactococci 62
4.1 *Lactococcus lactis* 1403...63
4.1.1 Growth of *L. lactis* 1403 in media containing valine supplied in different forms.......................................63
4.1.2 Peptidase activities of *L. lactis* 1403 grown on different nitrogen forms...66
4.1.3 Uptake of Val-$(Gly)_x$ peptides by *L. lactis* 1403................66
4.1.4 Assessment of leakage of intracellular enzymes from *L. lactis* 1403 cells...73
4.2 *Lactococcus cremoris* E_8...74
4.2.1 Growth and peptidase activities of *L. cremoris* E_8 in chemically defined medium.......................................74
4.2.2 Assessment of intracellular leakage from *L. cremoris* E_8 cells...76
4.2.3 Uptake of Val-$(Gly)_x$ peptides by *L. cremoris* E_8...............77
4.2.4 Utilisation of Val-$(Gly)_x$ peptides as a source of valine for growth of *L. cremoris* E_8 in chemically defined media...81
4.2.5 Energy dependence of Val-Gly_2 utilisation by *L. cremoris* E_8. 85

4.2.6 Assessment of the effect of potential competitors on Val-Gly transport. 87

4.2.7 Investigation of possible nutritional regulation of di-/ tripeptide transport in whole cells of *L. cremoris* E_8. 87

4.2.8 Preliminary studies into the utilisation of β-casein oligopeptides by *L. cremoris* E_8. 89

4.3 *Lactococcus lactis* 920. 93

4.3.1 Assessment of leakage of intracellular enzymes from *L. lactis* 920 cells. 93

4.3.2 Growth and peptidase activities of *L. lactis* 920 in chemically defined medium 94

4.3.3 Uptake of Val-$(Gly)_x$ peptides by *L. lactis* 920. 96

4.3.4 Utilisation of Val-$(Gly)_x$ peptides as a source of valine for growth of *L. lactis* 920 in chemically defined media. 99

4.3.5 Energy dependence of oligopeptide uptake in *L. lactis* 920 103

4.3.6 The effects of potential competitors upon Val-Gly uptake by *L. lactis* subsp. *lactis* 920 104

4.4 *Lactococcus cremoris* ML_3 108

4.4.1 Growth and peptidase activities of *L. cremoris* ML_3 on chemically defined media. 108

4.4.2 Assessment of leakage of intracellular enzymes from *L. cremoris* ML_3 cells. 108

4.4.3 Uptake of Val-$(Gly)_x$ peptides by *L. cremoris* ML_3. 108

4.4.4 The effects of potential competitors upon Val-Gly uptake by *L. lactis* subsp. *cremoris* ML_3 112

4.5 *Lactococcus cremoris* AM_2 112

4.5.1 Growth of *L. cremoris* AM_2 in chemically defined medium... 114

4.5.2 Uptake of Val-$(Gly)_x$ peptides by *L. cremoris* AM_2 114

4.6 *Lactococcus cremoris* SK_{11} 116

4.6.1 Growth of *L. cremoris* SK_{11} in chemically defined medium.. 116

4.6.2 Assessment of leakage of intracellular enzymes from *L. cremoris* SK_{11} cells. 119

4.6.3 Uptake of Val-$(Gly)_x$ peptides by *L. cremoris* SK_{11}. 120

4.7 Comparative study of the growth of *L. cremoris* strains on chemically defined medium. 121

Chapter 5: Attempts to use transport mutants in studies on peptide utilisation.......... 124

5.1 Characterisation of the product of L-alanyl-β-chloroalanine synthesis... 125

5.2 The attempted isolation of dipeptide transport mutants of *L. cremoris* E_8 using L-alanyl-β-chloroalanine 128

5.3 Peptide uptake by the oligopeptide transport mutant, *Lactococcus lactis* subsp. *lactis* KG301 129

Chapter 6: Discussion **131**

6.1 Peptide metabolism and the lactococcal proteolytic pathway 131

6.2 The validity of using whole-cell peptide uptake as a measure of peptide transport 134

6.3 Lactococcal oligopeptide transport and its possible role in the utilisation of β-casein oligopeptides. 136

6.4 Strain differences in the mechanisms of peptide utilisation 138

6.5 Regulation of peptide metabolism 147

6.6 Assessment of the importance of dipeptidase activity to peptide utilisation 148

6.7 Future directions for research on peptide metabolism in the lactococci 148

References **150**

LIST OF FIGURES

Figure 1.2.1.1 Major peptide products produced by digestion of (a) β-casein, (b) κ-casein by the cell envelope-associated proteinase from *L. cremoris* H_2 (a P_1 type proteinase-producing strain), and *L. cremoris* SK_{11} (a P_{111} type proteinase producing strain).....3

Figure 1.2.3.1 Possible topological models of the di-/tripeptide transport protein of *Lactococcus lactis*. (Reproduced from Hagting *et al*., 1994).....8

Figure 1.2.3.2 (a) Genetic organisation of the opp operon of *L. lactis* (b) Proposed model of the *L. lactis* oligopeptide system encoded for by the opp operon. (Reproduced from Kok and de Vos, 1994).....10

Figure 1.4.1.1 A possible organisation of the lactococcal proteolytic pathway.....12

Figure 1.4.1.2 An alternative organisation of the lactococcal proteolytic pathway.....14

Figure 2.2.4.1 A photograph and diagrammatic representation of the system used to grow lactococci in a 200 ml fermenter flask.....25

Figure 2.2.4.2 Demonstration of the necessity for two successive passages when preparing an inoculum for culture growth on chemically defined medium.....27

Figure 2.2.10.1 The effect of adding TFA to timed samples, either before the cells are removed, or after the cells are removed, on the measurement of Val-Gly_2 uptake by cell suspension of *L. cremoris* E_8.....38

Figure 2.2.11.1 Structure of L-alanyl-β-chloroalanine.....42

Figure 2.2.11.2 Diagrammatic representation of a method employed to use alanyl-β-chloroalanine to select for mutants deficient in their ability to transport and/or hydrolyse dipeptides.....44

Figure 3.1.1.1 Reverse phase HPLC profiles of (a) the crude peptide product resulting from the solution phase synthesis of Val-Gly_3, and (b) the purified product after passage through a C18 preparative column.....47

Figure 3.1.1.2 Mass spectrum of the purified product from Val-Gly_3 synthesis.....48

Figure 3.1.2.1 Reverse phase HPLC profiles of (a) the crude peptide product resulting from the solution phase synthesis of Val-Gly_4, and (b) the purified product after passage through a C18 preparative column.....50

Figure 3.1.2.2 Mass spectrum of the purified product from Val-Gly_4 synthesis.....51

Figure 3.1.2.3 Capillary electrophoresis profile of the purified product obtained from Val-Gly_4 synthesis.....52

Figure 3.1.3.1	Reverse phase HPLC profile of the purified product from the solid phase peptide synthesis of Val-Gly_7	54
Figure 3.1.3.2	Mass spectrum of the purified product from Val-Gly_7 synthesis	55
Figure 3.1.4.1	Reverse phase HPLC profile of the purified product from the solid phase peptide synthesis of Val-Gly_9	56
Figure 3.1.4.2	Mass spectrum of the purified product from Val-Gly_9 synthesis	57
Figure 3.2.1.1	Reverse phase HPLC profile of a 3 hour digest of β-casein by a purified extract of the cell envelope-associated proteinase from *L. cremoris* H_2	59
Figure 3.2.1.2	Alignment of the peptides produced from β-casein hydrolysis by a purified extract of the cell envelope-associated proteinase of *L. cremoris* H_2 against the known sequence of the C-terminal end of β-casein.	60
Figure 3.2.3.1	Capillary electrophoresis profiles of each of the three major peptides produced from β-casein hydrolysis by the cell wall-associated proteinase of *L. cremoris* H_2.	61
Figure 4.1.1.1	Growth of *L. lactis* subsp *lactis* 1403 in media containing 1.5% (w/v) lactose, and different nitrogen sources.	65
Figure 4.1.3.1	Capillary electrophoresis profiles from a time course study of Val-Gly_2 uptake by glycolysing cell suspensions of *L. lactis* subsp *lactis* 1403.	68
Figure 4.1.3.2	Graph showing the decrease in Val-Gly_2 concentration when this peptide was incubated with cell suspensions of *L. lactis* 1403, and in a subsequent incubation with the cell-free supernatant.	70
Figure 4.1.3.3	Summary of the procedure used for calculating the net rate of peptide uptake by whole cells	70
Figure 4.2.1.1	Growth of *L. lactis* subsp *cremoris* E_8 in chemically defined medium, in which all amino acids are supplied in their free state, and containing 1.5% (w/v) lactose.	75
Figure 4.2.3.1	Capillary electrophoresis profiles from a time course study of Val-Gly_2 uptake by glycolysing cell suspensions of *L. lactis* subsp *cremoris* E_8.	79
Figure 4.2.3.2	Capillary electrophoresis profiles from a 120 minute time course study of Val-Gly_7 uptake by glycolysing cell suspensions of *L. lactis* subsp *cremoris* E_8	80
Figure 4.2.4.1	Growth of *L. lactis* subsp *cremoris* E_8 in chemically defined media supplemented with 1.5% (w/v) lactose, and different valine sources.	84
Figure 4.2.6.1	Graphical representation of the data presented in Table 4.2.6.1	88
Figure 4.2.8.1	Graph showing the decrease in β-casein oligopeptides when incubated with cell suspensions of *L. cremoris* E_8	90
Figure 4.2.8.2	Capillary electrophoresis profiles from a time course study of peptide 1 (KAVPYPQ) uptake by glycolysing cell suspensions of *L. lactis* subsp *cremoris* E_8.	92

Figure 4.3.2.1	Growth of *L. lactis* subsp. *lactis* 920 in chemically defined medium, in which all amino acids are supplied in their free state, and containing 1.5% (w/v) lactose	95
Figure 4.3.3.1	Capillary electrophoresis profiles from a time course study of Val-Gly_7 uptake by glycolysing cell suspensions of *L. lactis* subsp *lactis* 920.	98
Figure 4.3.4.1	Growth of *L. lactis* subsp *lactis* 920 in chemically defined medium, containing 1.5% (w/v) lactose, and different valine sources	102
Figure 4.3.6.1	Graphical representation of the data presented in Table 4.3.6.1	106
Figure 4.4.1.1	Growth of *L. lactis* subsp *cremoris* ML_3 in chemically defined medium, in which all amino acids are supplied in their free state, and containing 1.5% (w/v) lactose	109
Figure 4.4.4.1	Graphical representation of the data presented in Table 4.4.4.1	113
Figure 4.6.1.1	Growth of *L. lactis* subsp *cremoris* SK_{11} in chemically defined medium, in which all amino acids are supplied in their free state, and containing 1.5% (w/v) lactose	117
Figure 4.6.1.2	Graphical representation of the data presented in Table 4.6.1.1	118
Figure 4.7.1.1	The growth of six *L. lactis* subsp *cremoris* strains through sequential passages in chemically defined medium containing free valine (CDM + Val)	122
Figure 5.1.1	Reverse phase HPLC profile of the crude peptide product resulting from the synthesis of L-alanyl-β-chloroalanine	126
Figure 5.1.2	Mass spectrum of peak (b) collected from an HPLC separation of the crude product from L-alanyl-β-chloroalanine synthesis	127
Figure 5.2.1	Mass spectrum of purified L-alanyl-β-chloroalanine collected from a subsequent HPLC separation of the crude product.	127
Figure 6.1.1	Diagrammatic representation of the processes and components involved in casein utilisation, indicating those which have been well characterised	132
Figure 6.3.1	A proposed model for the mechanism of utilisation of milk caseins by *L. lactis* subsp. *cremoris* E_8	139
Figure 6.4.1	The growth of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains on chemically defined medium in which all amino acids are supplied in their free state and containing 1.5% (w/v) lactose	140

LIST OF TABLES

Table 1.2.1.1 Classification of proteinases based on their activities towards caseins. 2

Table 1.2.2.1 A summary of the lactococcal peptidases characterised to date. 5

Table 2.2.1.1 Comparison of the amino acid composition of chemically defined media used by researchers for the growth of lactococci. 20

Table 2.2.10.1 Comparison of the experimentally determined capillary electrophoresis areas of 1 mM solutions of di-L-alanine, tri-L-alanine, tetra-L-alanine, penta-L-alanine and hexa-L-alanine, with their expected areas relative to that of di-L-alanine. 40

Table 3.1.1.1 Amino acid analysis of the purified product obtained from Val-Gly_3 synthesis. 48

Table 3.1.2.1 Amino acid analysis of the purified product obtained from Val-Gly_4 synthesis. 51

Table 3.1.3.1 Amino acid analysis of the purified product obtained from Val-Gly_7 synthesis. 55

Table 3.1.4.1 Amino acid analysis of the purified product obtained from Val-Gly_9 synthesis. 57

Table 3.2.1.1 Amino acid sequences of peptide detected within peaks from an HPLC separation of a β-casein hydrolysate produced by a purified extract of the cell envelope-associated proteinase of *L. cremoris* H2. 59

Table 4.1.1.1 Specific growth rates of *L. lactis* subsp. *lactis* 1403 on different media. 64

Table 4.1.2.1 Peptidase activities of *L. lactis* 1403 cells grown on different nitrogen sources.. 67

Table 4.1.3.1 Conversion of the capillary electrophoresis area data for Val-Gly_2 utilisation into actual concentrations. 69

Table 4.1.3.2 Net rates of uptake of Val-$(Gly)_x$ peptides by *L. lactis* subsp *lactis* 1403. 71

Table 4.1.4.1 Assessment of intracellular aldolase leakage from incubating whole cells of *L. lactis* subsp *lactis* 1403. 73

Table 4.2.1.1 Peptidase activities of a French Press extract of *L. cremoris* E_8 cells grown in chemically defined medium. 75

Table 4.2.2.1 Assessment of intracellular aldolase leakage from whole cells of *L. lactis* subsp *cremoris* E_8. 76

Table 4.2.3.1 Net rates of uptake of Val-$(Gly)_x$ peptides by *L. lactis* subsp. *cremoris* E_8. 78

Table 4.2.4.1	Determination of the growth rates of *L. lactis* subsp. *cremoris* E_8 on chemically defined media supplemented with different concentrations of the essential amino acid valine.	82
Table 4.2.4.2	Analytically determined valine concentrations in various chemically defined media used in growth experiments with *L. cremoris* E_8.	83
Table 4.2.5.1	Demonstration of the energy dependence of Val-Gly_2 uptake by *L. lactis* subsp. *cremoris* E_8.	86
Table 4.2.6.1	The effects of potential competitors on Val-Gly uptake by washed cell suspensions of *L. cremoris* E_8.	88
Table 4.2.7.1	The effect of different growth media upon the rate of transport of Val-Gly_2 by *L. lactis* subsp. *cremoris* E_8.	89
Table 4.3.1.1	Assessment of intracellular aldolase leakage from incubating whole cells of *L. lactis* subsp. *lactis* 920.	94
Table 4.3.2.1	Peptidase activities of a French Press extract of *L. lactis* 920 cells grown in chemically defined medium.	95
Table 4.3.3.1	Net rates of uptake of Val-$(Gly)_x$ peptides by *L. lactis* subsp. *lactis* 920.	97
Table 4.3.4.1	Determination of the growth rates of *L. lactis* subsp. *lactis* 920 on chemically defined media supplemented with different concentrations of the essential amino acid valine.	100
Table 4.3.4.2	Analytically determined valine concentrations in the various chemically defined media used in growth experiments with *L. lactis* 920.	101
Table 4.3.5.1	Demonstration of the energy dependence of Val-Gly_3 uptake by *L. lactis* subsp. *lactis* 920.	103
Table 4.3.5.2	The effect of the ATPase inhibitor vanadate on Val-Gly_3 uptake by whole cell suspensions of *L. lactis* subsp. *lactis* 920.	105
Table 4.3.6.1	The effects of potential competitors on Val-Gly uptake by washed cell suspensions of *L. lactis* subsp. *lactis* 920.	106
Table 4.4.1.1	Peptidase activities of a French Press extract of *L. cremoris* ML_3 grown in chemically defined medium.	109
Table 4.4.2.1	Assessment of intracellular aldolase leakage from incubating whole cells of *L. lactis* subsp. *cremoris* ML_3.	110
Table 4.4.3.1	Net rates of uptake of Val-$(Gly)_x$ peptides by *L. lactis* subsp. *cremoris* ML_3.	111
Table 4.4.3.2	Net rate of uptake of tetra-alanine by *L. lactis* subsp. *cremoris* ML_3.	111
Table 4.4.4.1	The effects of potential competitors on Val-Gly uptake by washed cell suspensions of *L. cremoris* ML_3.	113
Table 4.5.2.1	Net rates of uptake of Val-$(Gly)_x$ peptides by *L. lactis* subsp. *cremoris* AM_2.	115
Table 4.6.1.1	The effect of substrates upon the growth of *L. cremoris* SK_{11} in chemically defined medium.	118

Table 4.6.2.1	Assessment of intracellular aldolase leakage from incubating whole cells of *L. lactis* subsp. *cremoris* SK_{11}.	119
Table 5.3.1	Net rates of uptake of Val-$(Gly)_x$ peptides by *L. lactis* subsp. *lactis* KG301.	120
Table 6.4.1	Specific growth rates of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains on chemically defined medium	141
Table 6.4.2	Comparison of the peptidase activities of French Press extracts of *L. lactis* and *L. cremoris* strains grown in chemically defined medium	143
Table 6.4.3	Comparison of the net rates of transport of Val-$(Gly)_x$ peptides by *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains	144

Chapter One: Introduction

1.1 The importance of lactic acid starter bacteria in the dairy industry.

The conversion of lactose to lactic acid, and the degradation of casein micelles to produce a heterogeneous mixture of peptides and amino acids, are key biochemical events in the manufacture of fermented milk products such as yoghurt and cheese.

Of the various lactic acid bacteria capable of carrying out these enzymatic processes, it is members of the genus *Lactococcus*, particularly *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* that are employed preferentially in industry as starter cultures. (In future these subspecies will be referred to as *L. lactis* and *L. cremoris* respectively).

All lactococci are strictly fermentative, obtaining their ATP and reducing equivalents from the glycolytic metabolism of lactose to lactic acid. The production of lactate acidifies the milk to a final pH of 4 to 5. This acidification facilitates casein coagulation by dissolution of colloidal calcium phosphate in the micelles, and prevents the growth of undesirable micro-organisms in the resulting product.

Lactococcal bacteria have complex nutritional requirements. In addition to vitamins and nucleotides, they are unable to synthesise many of the amino acids required for growth (Reiter and Oram, 1962). *L. lactis* strains require fewer essential amino acids than *L. cremoris* strains (Smid, 1991).

Lactococci can exhibit exponential growth in milk, with a doubling time of 60 minutes at 30°C. Growth to high numbers over a short period is crucial to getting acid production at levels needed to form milk curds on a commercial scale (Laan *et al.*, 1989). Milk however, is a suboptimal medium for these fastidious bacteria, levels of free amino acids and usable peptides being well below their minimal requirement for protein synthesis (Thomas and Pritchard, 1987). More than 80 percent of milk nitrogen is locked up in the proteins α_{s1}, α_{s2}, β and κ-caseins.

The ability of lactococci to grow to high densities in milk is only possible because of a complex proteolytic system which enables them to rapidly degrade these caseins into metabolisable peptides and free amino acids.

1.2 The proteolytic system of lactococcal starter bacteria

The three essential components of the proteolytic system of starter bacteria are the proteinases, the peptidases and the transport systems.

1.2.1 Proteinases

These are cell wall associated serine proteases which catalyse the first step in milk protein degradation, namely the extracellular hydrolysis of caseins into oligopeptides of varying length.

In general these enzymes are plasmid encoded, show an optimum activity at pH 6.6, and have a size range of 80- 180 kDa depending on the method by which they are isolated from the cell (Pritchard and Coolbear, 1993).

The genetic determinants of these proteins are now well characterised. A pre-pro protein encoded for by the *prtP* gene undergoes post-translational modifications in association with a *prtM* gene product before insertion into the cell membrane. Sequence analysis of the *prtP* genes encoding proteinases from different lactococcal subspecies show them to be highly conserved (Kok, 1990, 1991).

On the basis of the specificity of casein hydrolysis, two types of proteinase are currently recognised (Exterkate and de Veer, 1989; Visser *et al.*, 1986). Details of these are summarised in Table 1.2.1.1

Table 1.2.1.1: Classification of proteinases based on their activities towards caseins.

PROTEINASE TYPE	SPECIFICITY	STRAINS
P_1 (HP type)	Major activity towards β–casein and minor activity towards κ-casein. No activity towards α_{s1}-casein	*L. cremoris* HP, WG_2, E_8* *L. lactis* NCDO606, NCDO607
P_{111} (AM_1 type)	Activity towards α_{s1}, β and κ-caseins	L. cremoris SK_{11}, AM_1, E_8*

* NB. E_8 shows a mixture of both proteinase types and so may represent an intermediate form.

These proteinase types differ in their time dependent-cleavage patterns of β and κ-caseins (Reid *et al.*, 1991a; 1994), as summarised in Figure 1.2.1.1.

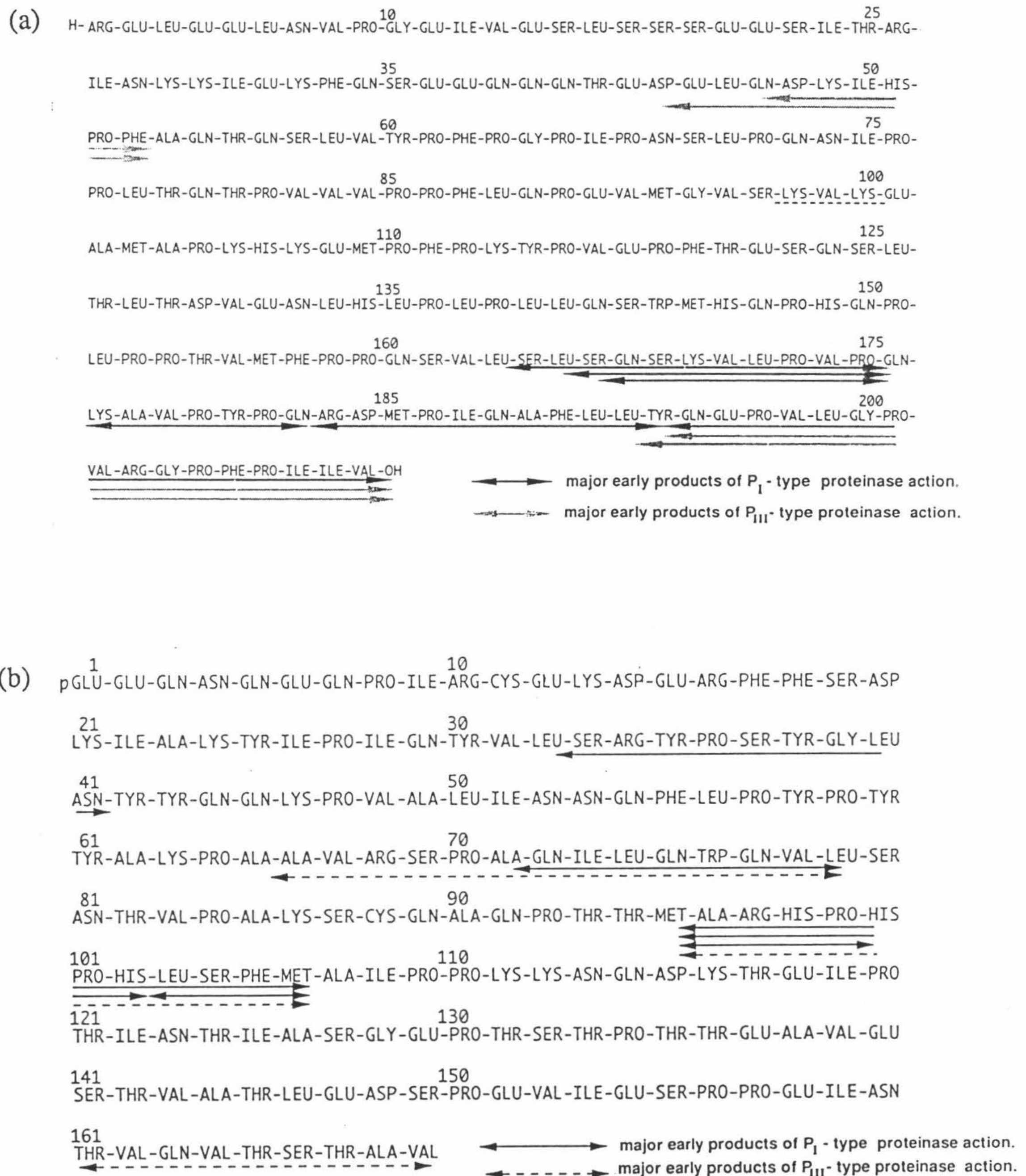

Figure 1.2.1.1 Major peptide products produced by digestion of (a) β–casein (b) κ-casein by the cell envelope-associated proteinase from *L. cremoris* H2 (a P_1-type proteinase-producing strain) and *L. cremoris* SK11 (a P_{111}-type proteinase producing strain).

During growth in milk, the lactococcal cells will be supplied with different oligopeptide products depending on whether they have a P_1 or P_{111} type proteinase.

Proteinase synthesis is regulated by both calcium levels in the medium and the nature of the nitrogen source upon which the cells grow. Calcium increases the level of proteinase activity while high levels of free amino acids or small peptides act instead to inhibit proteinase synthesis (Exterkate, 1985).

Recent studies on proteinase gene expression in genetically manipulated strains of lactococci containing multiple copies of the *prtP gene*, suggests that there are differences between strains in the stringency with which this gene is regulated (Bruinenburg *et al.*, 1992).Work with proteinase negative strains has shown these enzymes are essential to growth of lactococcal bacteria in milk.

1.2.2. Peptidases

Lactococci possess a large array of enzymes capable of degrading the oligopeptide products produced by the action of the cell wall-associated proteinase. A list of the peptidases isolated to date are presented in Table 1.2.2.1. While most of these enzymes have now been well characterised *in vitro*, several important questions remain unanswered about their activity *in vivo*.

One such question concerns the regulation of peptidase activity in response to differences in the composition of the growth medium. Law (1977) found that dipeptidase levels in *lactis* strains were lower when the cells were grown on amino acid-defined media than in yeast broth, suggesting that the presence of peptides leads to an increase in the activity of this peptidase.
However, van Boven *et al.* (1988) reported no observable differences in dipeptidase activity of the *lactis* strain Wg_2 when grown on different nitrogen sources. A comprehensive study is required to resolve these differences.

Another unresolved question is which of the various peptidase activities listed in Table 1.2 2.1 are essential for growth upon milk caseins. Answers to this question are best approached by the isolation of mutants deficient in a particular peptidase activity. Already such work using the highly specific technique of integrative gene inactivation (Leenhouts *et al.*, 1991) has demonstrated that neither endopeptidase PepO (Mierau *et al.*, 1993) nor X-prolyl dipeptidyl aminopeptidase (Mayo *et al.*, 1993) is essential for growth of *lactococci* in milk. Eventual identification of the essential enzyme complement for growth on casein is important for the development of improved industrial starters.

Table 1.2.2.1 A summary of the lactococcal peptidases characterised to date.

ENZYME	SPECIFICITY	REFERENCES
Dipeptidase	X---Y	Hwang *et al.*, 1981;1982. van Boven *et al.*, 1988
Tripeptidase (PepT).	X---YZ	Bosman *et al.*, 1990.
Endopeptidase LEPI LEPII PepO	...WX---YZ...	Yan *et al.*, 1987a Yan *et al.*, 1987b Tan *et al.*, 1991
Aminopeptidase A (PepA).	Glu---XY... Asp---XY...	Exterkate *et al.*, 1987 Niven, 1991
Aminopeptidase N (PepN)	W---XY...	Tan and Konings, 1990. Exterkate *et al.*, 1992 Tan *et al* ., 1992 Midwinter and Pritchard., 1994
Aminopeptidase C (PepC).	W---XY...	Neviani *et al* ., 1989 Chapot-Chartier *et al.*, 1992
Prolidase	X---Pro	Kaminogawa *et al.*, 1984 Booth *et al.*, 1990
Proiminopeptidase	Pro---X	Baankreis *et al.*, 1991 Zevaco *et al.*, 1990
X-prolyl dipeptidyl aminopeptidase	X---ProY	Keifer-Partsch *et al.*, 1989 Lloyd and Pritchard, 1991

Much debate also surrounds the cellular location of the peptidases, as will be discussed in Section 1.5.

1.2.3 Transport proteins

Research to date has identified three possible routes by which the products of extracellular protein and peptide degradation could enter the cell, namely as free amino acids, as di- or tripeptides, or as oligopeptides.

(i) Amino acid transporters.

These are integral membrane proteins which are highly selective for specific structural types of amino acids. Different bioenergetic mechanisms are employed in their function (Konings *et al.*, 1989).

(a) proton motive force linked transporters.

Couple transport of an amino acid to the movement of protons down their concentration gradient.
Examples: Val, Leu, and Ile by the branched chain amino acid transporter.
Ala, Gly and Ser by the neutral amino acid transporter

(b) antiporters/ exchange transporters

Couple the outward movement of a product down its concentration gradient with the inward movement of a substrate.
Example: arginine- ornithine antiporter in *L. lactis* strains.

(c) phosphate bond linked transporters

Couple amino acid transport directly to the hydrolysis of ATP or some other "high energy" phosphate bond.
Example: glutamate transport

As yet no genes for these transporters have been identified or characterised .

Expression of some amino acid transport systems appears to be regulated by peptides in the growth media (Poolman and Konings, 1988; Smid, 1991).
The arginine-ornithine antiporter has also been shown to be repressed when cells are grown in arginine-deficient media (Konings *et al.*, 1989).

(ii) Di-/tripeptide transport

The failure of tripeptides to inhibit Glycyl[^{14}C]leucine uptake in early studies with the *cremoris* strains E8 and 2016 suggested that separate proteins transported dipeptides and tripeptides (Law, 1978)

However it is now believed that one major system transports both peptide types. Strong evidence supporting this conclusion has come from the isolation of a mutant of the *cremoris* * strain ML3 resistant to the action of the toxic dipeptide L-alanyl-β-chloroalanine (AβClA). When cleaved, this peptide releases β-chloroalanine which blocks an alanine racemase essential to cell wall formation. Lactococci which can grow in its presence are deficient in their ability to either transport this peptide, or to hydrolyse it.
Careful physiological characterisation of this mutant showed it to be deficient in its ability to transport both dipeptides and tripeptides.
Tripeptide transport mutants resistant to the toxic tripeptide β-chloro-L-alanyl-L-alanyl-L-alanine are phenotypically identical to the AβClA mutants (Smid, 1991).

Competition studies with these mutants suggests that this transport protein has a broad substrate specificity, except for di- or tripeptides containing arginine. Free amino acids and oligopeptides are not transported by this protein.

Transport by this system is believed to be a two step process, peptide translocation across the cytoplasmic membrane by an integral membrane protein being followed by intracellular hydrolysis by peptidases (Smid *et al.*, 1989a).

Studies of membranes from the wild type ML3 strain fused with liposomes containing beef heart cytochrome c oxidase has shown transport to be an active process driven by the proton motive force (Smid *et al.*, 1989a).

The inability of di-/tripeptide transport mutants to grow in media where β-casein is the sole nitrogen source demonstrates that one or more essential amino acids from this milk protein must enter the cell only as a dipeptide or tripeptide. (Smid *et al.*, 1989b).

Di-/tripeptide transport is also important to meeting the proline demands of a rapidly growing starter culture. This amino acid makes up over 18 percent of β-casein

---

* ML3 was formerly regarded as a *lactis* strain. It now has been shown to be much more closely related to the *cremoris* strains (Godan et al., 1992).

It is essential to *L. cremoris* strains for growth, and stimulatory to *L. lactis* strains. There is no carrier protein for free proline and although passive diffusion does occur it does so at very slow rates. Proline containing peptides are however good substrates for this peptide transport system (Smid *et al.*, 1990).

Work with the *cremoris* strain E_8 grown on different nitrogen sources suggests that this transport system is constitutively expressed (van Boven *et al.*, 1988).

Recently the gene encoding this protein, *dtpT*, has been cloned and sequenced (Hagting *et al.*, 1994). The *dtpT* gene encodes for a 463 residue protein , which on the basis of topology studies, is believed to be composed of twelve membrane spanning helices (see Figure 1.2.3.1).

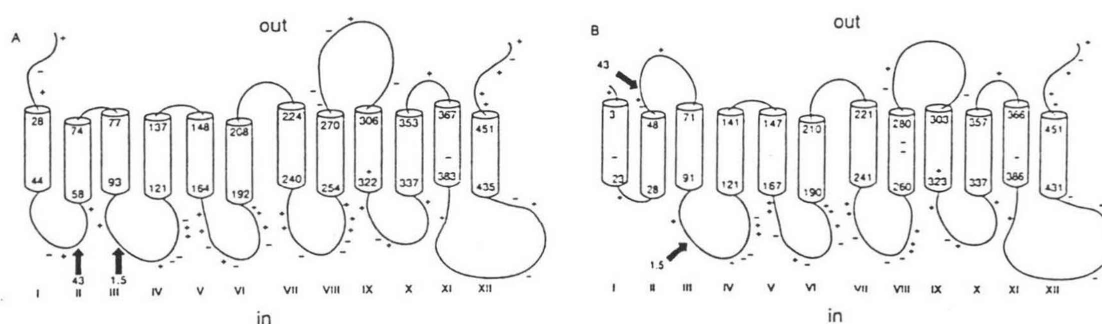

Figure 1.2.3.1 Possible topological models of the di-/tripeptide transport protein of *Lactococcus lactis* (Hagting *et al.*, 1994).

This protein has no significant homology with other bacterial peptide transport systems and so may represent a new type of transport protein.
Future work with the DtpT protein should provide clearer answers with respect to the mechanisms of transport and regulation of that process.

(iii) Oligopeptide transport

Various research supports the presence of a membrane carrier system for oligopeptides. The early work of Rice *et al.* (1978), and Law (1978) showed that oligopeptides can be used as an amino acid source. Apart from the results of

competition studies, little information was obtained about the mechanism by which such peptides were utilised.
For example, it was not known whether oligopeptides greater than three residues were transported intact , or only after hydrolysis into di -/tripeptides and amino acids

The isolation of *L. cremoris* ML3 mutants deficient in L-alanine, di-L-alanine and tri-L-alanine transport, but still capable of accumulating alanine when it was supplied as a tetrapeptide or pentapeptide suggested strongly that a separate transport system for oligopeptides did exist (Kunji *et al.*, 1993).

These results are supported by complementation studies. The introduction of a cloned DNA fragment from *L. lactis* strain SSL135 into a proteinase-positive strain not capable of growth in milk , enabled this mutant to grow on tryptic peptides of casein (Tynkkynen *et al.*, 1993). Work with the DNA fragment in *Escherischia coli* cells showed that it encoded an oligopeptide transport system (Tynkkynen *et al.*, 1993).

Whereas the di-/tripeptide transport system is encoded for by one gene, the oligopeptide transport system appears to be encoded for by an operon of five genes - *opp* DFBCA (Tynkkynen *et al.*, 1993). The proteins produced from the *opp* DFBCA operon constitute a system that is characteristic of the ATP-binding cassette family of transport proteins (see Figure 1.2.3.2). Oligopeptide transport is an active process which occurs in the absence of electrochemical gradients and can be inhibited by ATPase inhibitors such as vanadate. These results suggest that transport is coupled to hydrolysis of ATP (Kunji *et al.*, 1993).

Work with *cremoris* ML3 mutants suggests that this protein has a narrow substrate specificity. Oligopeptides containing prolyl, glutamyl, aspartyl or arginyl residues are not substrates for this transport protein (Smid, 1991).

Tetraalanine uptake in these mutants was also found to occur at a much slower rate than dialanyl or trialanyl uptake, indicating this system to be significantly less active than the di-tripeptide transport system. These results are supported by the earlier studies of Rice *et al.* (1978). They found that rates of $Gly_3[^{14}C]$Leu-Gly and $Gly_2[^{14}C]$Leu-Gly uptake by the cremoris strain C_{10} to be one-seventh and one-fifth the rate of Gly-$[^{14}C]$Leu uptake.

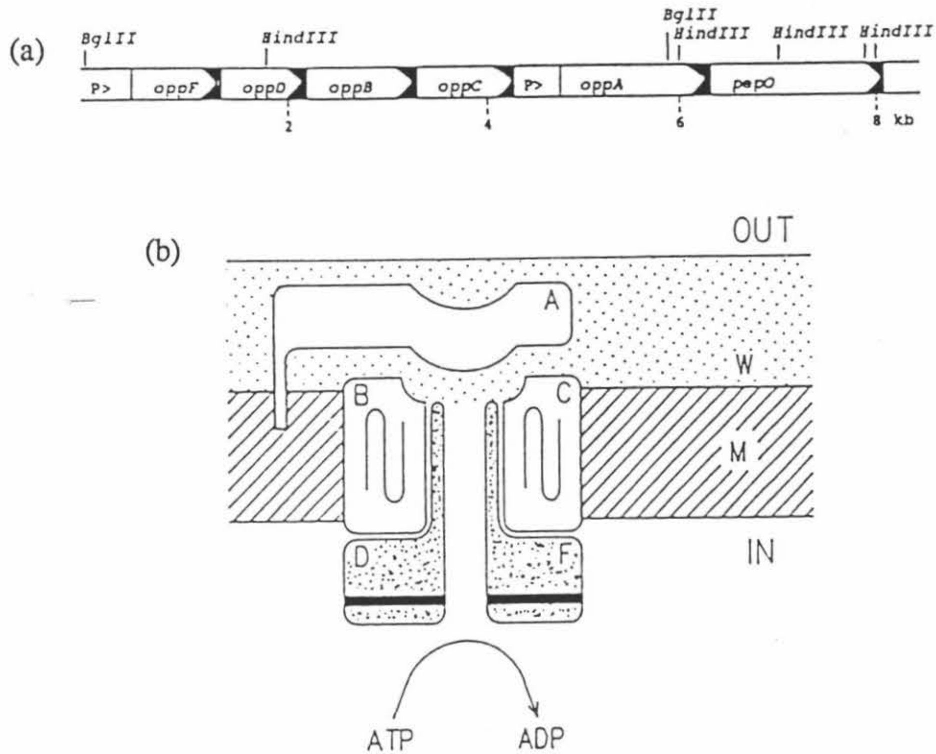

Figure 1.2.3.2

(a) Genetic organisation of the *opp* operon of *L. lactis*

(b) Proposed model of the *L. lactis* oligopeptide system encoded for by the *opp* operon (Kok and de Vos, 1994).

The inability of other mutants of strain ML_3, in which either *opp A* or the entire operon were deleted, to grow in milk demonstrated that this transport system was essential to casein utilisation (Tynkkynen *et al.*, 1993; Yu , 1994).

Initial findings also suggested that the activity of this transport system was dependent upon the nature of the nitrogen source present in the medium (Kunji *et al.*, 1993).

1.3 Peptide metabolism and its significance.

Peptide metabolism can be viewed as those processes involved in the transformation of casein-derived oligopeptides into an intracellular pool of metabolisable amino acids. This is a significant area of research for two principle reasons.

(a) Lactococcal nutrition.

Although proteinase activity is essential to the degradation of milk proteins, further metabolism of the oligopeptides to free amino acids is essential to supply the amino acid requirements for growth because of the low amount of free amino acids available in milk.

Supplying milk-grown, proteinase positive and proteinase negative strains with small peptides and free amino acids improves both growth rates and yields (Thomas and Mills, 1981).The maximum growth rates attained when lactococci are grown in media containing peptides as sources of essential amino acids are higher than those in media containing amino acids alone (Hugenholtz *et al.*, 1987).

A comprehensive understanding of peptide metabolism is therefore essential for success in the pursuit of faster growing strains for industry.

(b) Product quality.

Peptide metabolism influences the flavour and texture of cheeses. Distinct flavour characteristics such as bitterness are a consequence of the accumulation of peptides not utilised by starter bacteria during both the initial period of cell growth and while the cheese is ripening (Stadhouders *et al.*, 1983; Visser *et al.*, 1983).

Certain strains characteristically produce savoury cheeses while others typically produce bitter ones. This implies important differences in the mechanisms by which these strains metabolise milk peptides. An understanding of these differences should enable us to manipulate starter cultures to produce cheeses with consistently favourable organoleptic properties.

1.4 The role of peptide metabolism in the lactococcal proteolytic pathway.

While peptide metabolism encompasses the action of both peptidases and transport systems, the relative contribution of each to the utilisation of casein peptides is much debated.

1.4.1 Current models of peptide metabolism

Two models can be proposed from the research available to date.

(a) Extracellular cleavage precedes transport.

In this model extracellular peptidases act to degrade the oligopeptides produced by the cell wall-associated proteinase into smaller peptides, principally di-/tripeptides, and free amino acids (see Figure 1.4.1.1).

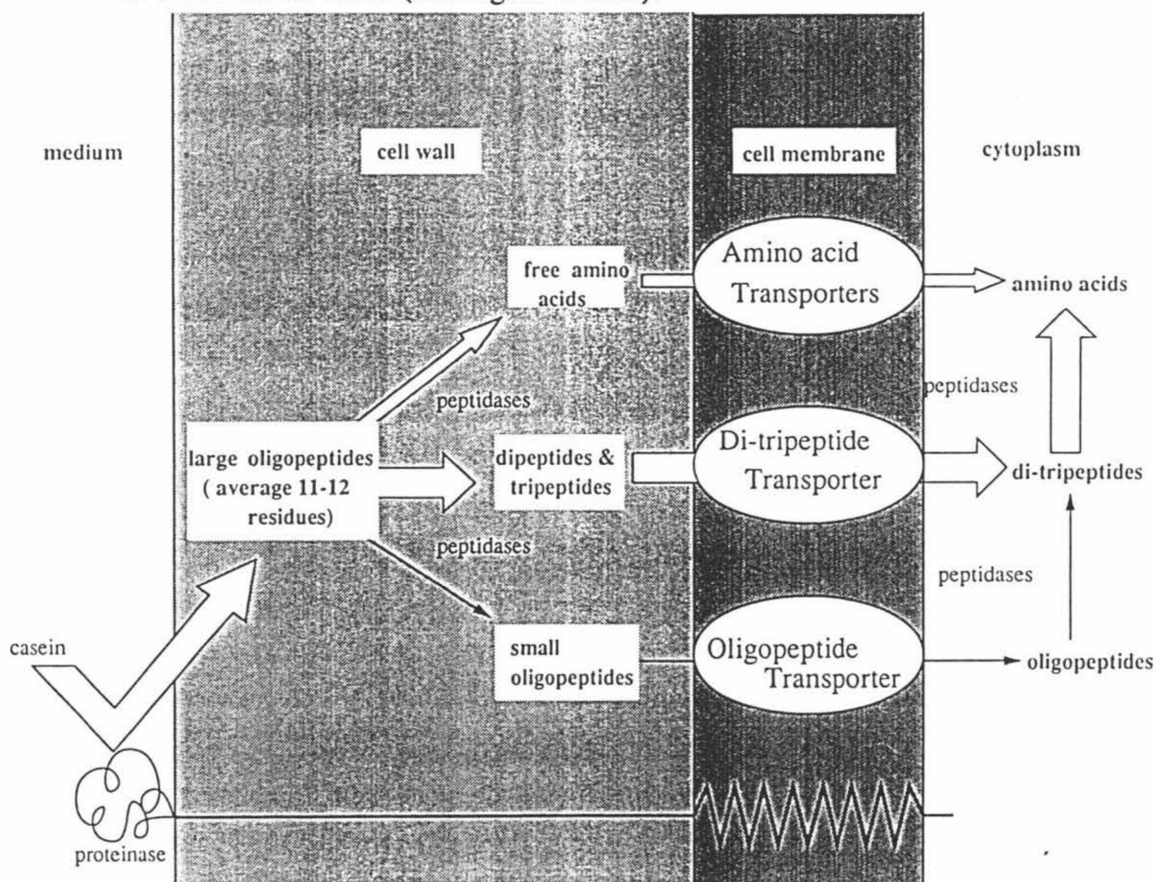

Figure 1.4.1.1: A possible organisation of the lactococcal proteolytic pathway. Current evidence indicating low relative rates of activity of the oligopeptide transport system (Smid, 1991; Kunji *et al.*, 1993), support this model which favours a predominant role for the di-/tripeptide transport system

It has been predicted from knowledge of the type of oligopeptides produced by the cell wall-associated proteinases, that the extracellular peptidase complement would have to involve at least a general aminopeptidase, an X-prolyl dipeptidyl aminopeptidase, and a glutamyl aminopeptidase (Smid, 1991).

Other studies have implicated an extracellular presence for a dipeptidase (Kolstad and Law, 1985), a tripeptidase (Bacon *et al.*, 1993; Sahlstrom *et al.*, 1993), a general aminopeptidase (Exterkate, 1984), an aminopeptidase A (Exterkate and de Veer 1987a), and an endopeptidase (Yan *et al.*, 1987a; O'Harte *et al.*, 1993).

However, the results of many of these studies are not conclusive, a major cause of uncertainty lying in the difficulty of obtaining cell fractions free from intracellular enzyme contamination. For example, the "cell wall" associated dipeptidase identified by Kolstad and Law (1985) was found to have specificity profiles very similar to that of its intracellular counterpart.

While it is possible that some extracellular peptidase activity could result from cellular leakage itself, this is unlikely to become significant until the later stages of growth when cell numbers are already at high densities.

The recent isolation and sequencing of genes encoding lactococcal peptidases has provided evidence against an extracellular location for peptidase activity, as none of the peptidases characterised to date has the leader sequence characteristic of extracellular targeted proteins (Kok and de Vos, 1994).

(b) Transport precedes intracellular cleavage.

In this model the products of proteinase-catalysed casein degradation are transported directly into the cell after which they are rapidly degraded by internal peptidases (see Figure 1.4.1.2).

However, the demonstration that the di-/tripeptide carrier system is essential for growth of the *cremoris* strain ML_3, necessitates a more extensive cleavage of β–casein than is currently known to be the case (Smid *et al.*, 1989b).

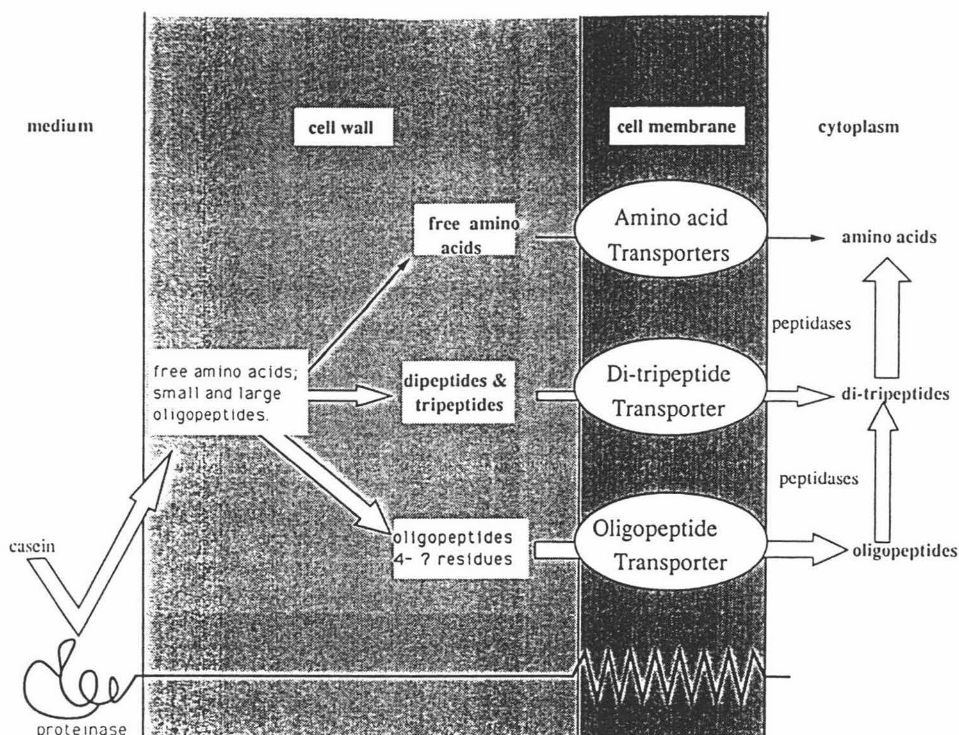

Figure 1.4.1.2. An alternative organisation of the lactococcal proteolytic pathway.

Current evidence on the identity of the peptide products of casein hydrolysis by the cell wall-associated proteinase (Monnet *et al.*, 1989; Visser *et al.*, 1988, 1991, 1994; Reid *et al.*, 1991a, 1991b, 1994) indicate that the minimum size of these peptides is 6 to 7 amino acid residues. However, it is possible that smaller products are not being detected by the analytical techniques used. Alternatively, small peptides may not be produced by the proteinase when it is released from the cell wall for *in vitro* studies.
If all the peptidases are eventually shown to have an intracellular location, then the extent to which lactococcal proteinases can degrade casein will be an area requiring extensive research.

This model implies a predominant role for the oligopeptide transport system. Current knowledge (Smid, 1991; Kunji *et al.*, 1993) does not suggest that this system is capable of transporting a sufficient range of larger peptides at a rate which would support rapid growth. However these conclusions come from a small number of studies involving only a single lactococcal strain. Furthermore the results of these are in conflict with other earlier research done in this area as discussed previously.

More information is therefore needed about this transport system particularly regarding the upper size limit of peptides it will transport, substrate specificity and the relative rates of peptide transport.

1.4.2 Strain Differences in Peptide Metabolism.

Most of the work carried out on peptide transport systems has come from the studies in Koning's laboratory in Groningen on the *cremoris* strain ML_3 and its mutants. While these have provided us with valuable information, they have not questioned whether the ML_3 strain is "typical" of the major cheese producing strains.

Law (1977) found that there were actually three quite distinct groups with regard to peptide utilisation.

(1) Those *lactococci* that transport dipeptides prior to hydrolysis ie the dipeptides are transported as such.

(2) Those *lactococci* that transport the amino acid constituents after dipeptide hydrolysis

(3) Those *lactococci* that did not transport dipeptides at all.

This raises important questions about the validity of applying the knowledge obtained from the ML_3 strain to the *lactococci* as a group.

1.5 Aims of the Current Study

The central objective of this research is to investigate aspects of peptide utilisation by whole lactococcal cells. The results from this work will hopefully provide answers to some important questions in this area.

(1) Is the oligopeptide transport system capable of transporting large peptides and at physiologically relevant rates ?

(2) Are there any significant differences in the process of peptide utilisation between the ML3 strain and other strains ?

(3) What peptidases are essential for growth ?

(4) What factors regulate the enzyme component of peptide utilisation?

An attempt to answer some of these questions will involve the following experimental strategies:

(a) The synthesis of a homologous series of peptides of increasing size from the dipeptide Val-Gly to the decapeptide Val-Gly9. Valine was chosen as the amino acid on which to base this series as is essential to the growth of both *lactis* and *cremoris* strains (Reiter and Oram, 1962).

(b) Peptide uptake studies with this series of peptides by the *cremoris* strains ML3, E8, and SK11, and the *lactis* strains 1403 and 920.

(c) Growth studies with these same strains in chemically defined media, where free valine is substituted for by the Val-(Gly)$_x$ series of peptides.

(d) Peptide uptake studies using oligopeptides isolated by HPLC from a proteinase digest of β-casein.

(e) The isolation and characterisation of mutants deficient in peptide utilisation from one lactococcal strain.

(f) The investigation of the effect of nitrogen source type upon the expression and activity of intracellular peptidases.