



UNIVERSITI PUTRA MALAYSIA

**DEVELOPMENT OF AN ANCHORING SYSTEM FOR PROTEIN
DISPLAY ON THE CELL WALL SURFACE OF LACTOCOCCUS
LACTIS MG1363**

NADIMPALLI RAVI SANKARA VARMA.

FBSB 2006 9



**DEVELOPMENT OF AN ANCHORING SYSTEM FOR PROTEIN DISPLAY ON
THE CELL WALL SURFACE OF *LACTOCOCCUS LACTIS* MG1363**

By

NADIMPALLI RAVI SANKARA VARMA

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfilment of the Requirement for the Degree of Doctor of Philosophy**

March 2006



To my Gurus, parents and wife



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

**DEVELOPMENT OF AN ANCHORING SYSTEM FOR PROTEIN DISPLAY
ON THE CELL WALL SURFACE OF *LACTOCOCCUS LACTIS* MG1363**

By

NADIMPALLI RAVI SANKARA VARMA

March 2006

Chairman : Associate Professor Raha Abdul Rahim, PhD

Faculty : Biotechnology and Biomolecular Sciences

Lactococcus is one of the lactic acid bacteria that are widely used in various food and fermentation processes. They have been used for many centuries in food fermentation processes and are considered as GRAS organisms that can safely be used in medical and veterinarian applications. The anchoring of proteins to the cell surface of *Lactococcus* using recombinant DNA techniques is an exciting and emerging research area that holds great promise for a wide variety of biotechnological applications. Presently available anchoring systems are based on recombinant bacteria displaying proteins or peptides on the cell surface. The objectives of this study are to develop surface display vectors and study the display of recombinant proteins on the surface of *Lactococcus lactis*.

Several anchor proteins have been identified in *L. lactis*. In this study the gene coding for the cell wall binding domain of *L. lactis* cell wall anchor proteins AcmA and NisP were amplified by PCR and cloned into an *E. coli* expression vector. Sequencing results showed 98% homology to published sequences. The plasmids designated as pSVacm and pSVnp were then transformed into *E. coli* where SDS-PAGE and Western blot



analyses showed that the cell wall binding domain of *acmA* and *nisP* genes were successfully expressed at the expected sizes 15 kDa and 18 kDa respectively. After mixing of the purified recombinant AcmA and NisP proteins with *L. lactis* cells, their presence on the bacteria cell surface was observed by whole cell ELISA, Ni²⁺ binding and fluorescence microscopy analysis.

The stability assay indicates that the binding of AcmA protein to the lactococcal cell surface was stable and can be retained on the cell wall surface for at least 5 days. The results from the pH study indicated that low pH had no significant effect on the stability of bound His-tag AcmA protein. Whilst the cell wall binding domain of AcmA was shown to be able to anchor to the cell surface of other Gram-positive bacteria tested in this study, AcmA protein was not able to bind to the surface of *E. coli* (Gram-negative) cells. Studies were also carried out to enhance the binding of AcmA protein to *L. lactis* cells where pretreatment of *L. lactis* with 10% TCA was shown to improve binding of the AcmA protein.

The new method developed for cell surface display of recombinant proteins on *L. lactis* was evaluated for expression and display of foreign proteins. The gene coding for the N-terminal epitope regions (VP1_{1-67aa} and VP1_{35-100aa}) of VP1 protein of Enterovirus 71 (EV71) were subcloned upstream to the cell wall binding domains sequences of plasmids pSVacm and pSVnp. SDS-PAGE and Western blot results confirmed the expression of N-terminal regions of VP1 protein as AcmA and NisP fusion proteins in *E. coli*. Whole-cell ELISA and immunofluorescence microscopy assays showed the successful display of VP1 protein of EV71 on the surface of *L. lactis*. The success of

docking VP1_{1-67aa} and VP1_{35-100aa} epitopes of VP1 on the surface of *L. lactis* cells using the anchoring system developed in this study, open up the possibilities of peptide and protein display for not only *Lactococcus* but of other Gram-positive bacteria. Preliminary studies showed that mice immunized with *L. lactis* displaying VP1_{1-67aa} or VP1_{35-100aa} fusion proteins were able to induce an immune response against the VP1_{1-67aa} or VP1_{35-100aa} (antigens). The new method developed for surface display has the potential to a variety of applications including screening of polypeptide libraries, development of live vaccines, construction of whole cell allosteric biosensors, and signal transduction studies.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PERKEMBANGAN SISTEM PELEKATAN BAGI PAMERAN PROTEIN PADA PERMUKAAN DINDING SEL *LACTOCOCCUS LACTIS* MG1363

Oleh

NADIMPALLI RAVI SANKARA VARMA

Mac 2006

Pengerusi : Profesor Madya Raha Abdul Rahim, PhD

Fakulti : Bioteknologi dan Sains Biomolecul

Lactococcus adalah sejenis bakteria asid laktik yang telah digunakan dengan meluas dalam pelbagai jenis makanan dan proses penapaian. *Lactococcus lactis* mempunyai beberapa sifat yang terpilih sebagai medium bagi penghantaran sebatian-sebatian yang mempunyai kepentingan farmaseutikal ke mukosa. Ia telah digunakan berabad-abad lamanya dalam proses penapaian makanan dan dikenali sebagai organisma GRAS yang selamat digunakan dalam aplikasi perubatan dan veterinar. Pelekatan protein pada permukaan sel *Lactococcus* menggunakan teknik rekombinan DNA merupakan suatu bidang penyelidikan yang menarik serta menjanjikan harapan yang tinggi kepada pelbagai penggunaan bioteknologi.

Beberapa protein pelekatan telah dikenalpasti dalam *L. lactis*. Dalam kajian ini, domain pengikatan dinding sel bagi dua protein pelekatan dari *L.lactis* iaitu gen *acmA* dan *nisP* telah diampifikasi melalui PCR. Keptu serpihan gen *acmA* dan *nisP* yang telah diampifikasi melalui PCR berjaya diklonkan ke dalam vektor penzahiran *E.coli* analisis jujukan DNA menunjukkan bahawa gen yang diklonkan adalah 98% homologi



dengan jujukan yang telah diterbitkan. Analisis SDS-PAGE dan “western blotting” mengesahkan bahawa domain pengikatan dinding sel bagi gen *acmA* dan *nisP* telah berjaya dizahirkan di dalam *E. coli* BL21(DE3)pLysS dan protein-protein rekombinannya berpadanan dengan saiz jangkaan iaitu 15 kDa dan 18 kDa masing-masing. Protein AcmA dan NisP yang tulen dicampurkan dengan sel-sel *Lactococcus* dan keputusan ELISA menunjukkan bahawa kedua-dua protein tersebut telah berjaya melekat pada permukaan sel *L. lactis*. Mikroskopi konfokal dan florsen mengesahkan keputusan tersebut. Asei kestabilan menunjukkan bahawa pelekatan protein AcmA pada permukaan sel *Lactococcus* adalah stabil dan boleh dikekalkan pada dinding sel selama sekurang-kurangnya lima hari. Di samping itu, domain pengikatan dinding sel AcmA telah ditunjukkan berupaya untuk melekat pada permukaan sel bakteria gram-positif lain yang diuji dalam kajian ini. Walau bagaimanapun, protein AcmA tidak boleh melekat pada permukaan sel *E. coli*. Sistem baru yang direka bagi pelekatan protein rekombinan pada permukaan sel *L. lactis* ini telah dinilai bagi penzahiran dan pelekatan bahagian VP1_{1-67aa} dan VP1_{35-100aa} bagi protein VP1 dari EV71. Bahagian VP1_{1-67aa} dan VP1_{35-100aa} bagi protein VP1 dari EV71 ini telah diklon sebelum domain pengikatan dinding sel protein AcmA dan NisP. Keputusan SDS-PAGE dan “western blotting” mengesahkan penzahiran bahagian VP1_{1-67aa} dan VP1_{35-100aa} bagi protein VP1 sebagai protein gabungan AcmA dan NisP. Asei-asei ELISA seluruh-sel dan mikroskopi imunoberpendarfluor menunjukkan kejayaan pelekatan protein VP1 dari EV71 pada permukaan *L. lactis*. Kejayaan pelekatan epitop-epitop VP1_{1-67aa} dan VP1_{35-100aa} bagi VP1 pada permukaan sel *L. lactis* dengan menggunakan sistem pelekatan yang dikembangkan dalam kajian ini membuka peluang bagi pelekatan peptida dan protein pada bukan sahaja *lactococcus* tetapi juga bakteria gram-positif yang lain.

Kajian ini menunjukkan bahawa *L. lactis* berputensi sebagai satu vaksin oral yang mempunyai peptida-peptida melekat pada permukaan sel nya serta mudah untuk menentukan kepekatan protein atau peptida yang akan diperkenalkan. Kajian ini menunjukkan bahawa pelekatan protein VP1 dari EV71 pada permukaan sel *L. lactis* boleh digunakan dalam perkembangan vaksin bukan-rekombinan hidup. Sistem baru pelekatan pada permukaan ini boleh digunakan dalam pelbagai jenis aplikasi, termasuk penyaringan perpustakaan polipeptida, perkembangan vaksin hidup, pembinaan bioderia, dan kajian transduksi isyarat.

ACKNOWLEDGEMENTS

I would like to express my wholehearted indebtedness to my major advisor, Associate Professor Dr. Raha Abdul Rahim for her inestimable and propitious guidance throughout the course of research and in transcription of this thesis. She has been an excellent to discuss with, and due to her extensive knowledge in the fields of Molecular Biology, many pieces of information have been found without having to look any further. Her care throughout my stay in Malaysia is greatly acknowledged. I express special thanks to my advisor for giving me an opportunity to do Ph.D. in Malaysia.

I am deeply grateful to Professor Dr. Khatijah Mohd Yusoff my co-supervisor, for her invaluable guidance and constant encouragement has helped me a lot during the course of study. Whenever there has been uncertainty of how to perform the experiments, she has had the time and her experience to share. My heart full thanks to Associate Professor Dr. Foo Hooi Ling, member of my advisory committee for her invaluable and kind suggestions and encouragements throughout the period of research. In addition, she has given me a number of ideas to consider when writing the thesis and not to forget her invaluable work in correcting my English. I am deeply grateful to my supervisors for guiding me into the field of surface display. I am indebted to them for engaging me in surface display, a subject caught my interest for good. Their positive attitude, encouragement and faith in my work have been great importance to me.



My heartfelt thanks to Yiap, Ernie, Hossein, Wei Yeng, Chyan Leong, Li Lung, Hooi Ling, Sing King, Yanti, and all members of Microbial Molecular Biology Laboratory, genetics lab and ATCL, for their moral support, advice and encouragement in the course of my study. I am grateful to all the people in the lab for making it a cosy and helpful place to work at.

I am thankful to all my lecturers who have laid the path of my educational career. I owe all my success to my parents for their blessing and love they have been showing and making me what I am. My heartfelt thanks to my family members for giving me the support that I needed, and for making me fly instead of diving. I am thankful to my friends and housemates for their blessings and support throughout my studies. I thank heartily Universiti Putra Malaysia for giving me this opportunity to pursue my Ph.D. in Malaysia. Finally, with all my heart I would like to thank my wife for her love and care.

TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vi
ACKNOWLEDGEMENTS	ix
APPROVAL	xi
DECLARATION	xiii
LIST OF TABLES	xix
LIST OF FIGURES	xx
LIST OF ABBREVIATIONS	xxv
CHAPTER	
I INTRODUCTION	1
1.1 Introduction	1
1.2 Objectives	5
II LITERATURE REVIEW	6
2.1 Lactic acid bacteria	6
2.2 Classification and physiological characteristics	7
2.3 LAB as constituents of the intestinal microflora	9
2.4 Adhesion of LAB to mucus	10
2.5 Cell wall composition	12
2.6 Peptidoglycan hydrolases of Gram-positive bacteria	17
2.7 Properties and growth of <i>Lactococcus lactis</i>	18
2.8 Surface display systems	20
2.8.1 Common features of anchored surface proteins	21
2.8.2 Surface display systems developed for Gram-negative bacteria	25
2.8.3 Surface display systems developed for yeast	26
2.8.4 Phage display systems	27
2.8.5 Surface display in gram-positive bacteria	28
2.9 Surface display in <i>Lactococcus</i>	31
2.9.1 The <i>N</i> -acetylmuramidase of <i>L. lactis</i>	31
2.9.2 PrtP	35
2.9.3 NisP (serine protease) of <i>Lactococcus</i>	36
2.9.4 HtrA	39
2.10 Cell location of heterologous proteins in <i>L. lactis</i>	39
2.11 Lactic acid bacteria as carriers of foreign molecules	41
2.12 <i>Lactococcus</i> as delivery system	42
2.13 Applications of surface display technology	47
2.13.1 Biosorbents for heavy metal removal	47
2.13.2 Detoxification of organic contaminants	49



2.13.3	Cell surface display of enzymes	49
2.13.4	Peptide library screening	49
2.13.5	Cell Surface display of antibody fragments	50
2.13.6	Production of anti-peptide antibodies	50
2.14	Overview of <i>E. coli</i> expression system used in this study	51
2.14.1	<i>E. coli</i> BL21(DE3)pLysS	51
2.14.2	pRSET expression vector	52
2.15	Enterovirus 71 (EV71)	52
2.15.1	Genome organization of EV71	55

III	DEVELOPMENT OF EXPRESSION VECTORS BASED ON THE CELL WALL BINDING DOMAINS OF N-ACETYLMURAMINIDASE AND SERINE PROTEASE	58
3.1	Introduction	58
3.2	Materials and methods	60
3.2.1	Bacterial strains and plasmids	60
3.2.2	Preparation of Stock Cultures	60
3.2.3	Extraction of Genomic DNA	61
3.2.4	Quantification of DNA concentration	62
3.2.5	Preparation of competent cells <i>E. coli</i>	62
3.2.6	Plasmid isolation from <i>E. coli</i> by modified alkaline lysis method	63
3.2.7	DNA gel electrophoresis	64
3.2.8	Restriction and ligation	65
3.2.9	Polymerase Chain Reaction	66
3.2.10	Purification of the PCR Product	67
3.2.11	Cloning of Lactococcal cell wall binding genes into <i>E. coli</i> expression vector	68
3.2.11.1	Cloning of <i>acmA</i> gene into pRSETC	68
3.2.11.2	Cloning of <i>nisP</i> gene into pRSETC	70
3.2.12	Transformation of <i>E. coli</i> (BL21(DE3)pLysS)	72
3.2.13	Screening of transformants	73
3.2.14	Verification of recombinant plasmid DNA	74
3.2.15	Sequencing of Recombinants (pSV _{acm} , pSV _{np})	75
3.2.15.1	DNA Sequencing Analysis	75
3.3	Results	76
3.3.1	Amplification of cell wall binding domain of <i>acmA</i> and <i>nisP</i> gene	76
3.3.2	Cloning of the cell wall binding domain of <i>acmA</i> and <i>nisP</i> genes into pRSETC vector	79
3.3.3	Verification of Recombinant pSV _{acm} and pSV _{np}	79
3.3.4	Sequence analysis of recombinant plasmids	85
3.3.5	Homology comparisons between cell binding domain of <i>acmA</i> gene and cell wall hydrolyases of other gram-positive bacteria	85

3.4	Discussion	91
-----	------------	----

IV	EXPRESSION AND BINDING STUDY OF CELL WALL BINDING DOMAINS OF THE RECOMBINANT <i>N</i>-ACETYLMURAMIDASE AND SERINE PROTEASE	96
4.1	Introduction	96
4.2	Materials and methods	97
4.2.1	Bacterial strains and plasmids	97
4.2.2	Expression of cell wall binding domains of AcmA and NisP proteins in <i>E. coli</i>	97
4.2.3	Protein Analysis	98
4.2.3.1	Sample preparation	98
4.2.3.2	SDS-PAGE Gel preparation	98
4.2.3.3	Western Blotting	100
4.2.4	Purification of the recombinant proteins	101
4.2.5	Quantification of recombinant proteins	101
4.2.6	Binding Analysis	102
4.2.7	Binding of cell wall binding domains of AcmA and NisP proteins to lactococcal cells	102
4.2.8	Immunofluorescence staining	103
4.2.9.1	Preparation of Poly-L-lysine coated slides	103
4.2.9.2	Immunofluorescence	103
4.2.9	Enzyme-linked immunosorbant assay (ELISA)	104
4.2.10	Whole cell Ni ²⁺ -binding assay	106
4.3	Results	106
4.3.1	Analysis of expression of cell wall binding domain of <i>acmA</i> and <i>nisP</i> genes	107
4.3.1.1	Expression analysis of cell wall binding domain of <i>acmA</i> gene	107
4.3.1.2	Expression analysis of cell wall binding domain of <i>nisP</i> gene	107
4.3.2	Purification of the Recombinant proteins	109
4.3.3	The study of binding ability of cell wall binding domain of AcmA protein onto the surface of <i>L. lactis</i>	112
4.3.3.1	Whole-cell ELISA analysis	112
4.3.3.3	Whole-cell Ni ²⁺ binding assay	114
4.3.3.4	Binding analysis by immunofluorescence	114
4.3.4	The binding ability of cell wall binding domain of NisP protein on the surface of <i>L. lactis</i>	118
4.3.4.1	Whole cell ELISA analysis	118
4.3.4.2	Whole cell Ni ²⁺ binding assay	118
4.3.4.3	Immunofluorescence microscopy	120
4.3.5	Study of binding ability of cell wall binding domain of AcmA protein to other gram-positive bacteria	124
4.3.6	Study of binding ability of cell wall binding domain	



	of AcmA protein to <i>E. coli</i>	124
4.4	Discussion	127
V	STABILITY AND STORAGE STUDY OF CELL WALL BINDING DOMAIN OF N-ACETYLMURAMIDASE	134
5.1	Introduction	134
5.2	Materials and methods	135
	5.2.1 Bacterial strains and plasmids	135
	5.2.2 Binding of cell wall binding domains of AcmA to lactococcal cells	135
	5.2.3 Pretreatment of <i>L. lactis</i> cells with 10% TCA	136
	5.2.4 Enzyme-linked immunosorbant Assay (ELISA)	136
	5.2.5 Stability assay	137
	5.2.6 Binding stability of surface display proteins at different pH environments	138
	5.2.7 Binding stability determination by treatment of <i>L. lactis</i> displaying AcmA with 8 M LiCl.	139
	5.2.8 Effect of pH on cell wall binding of AcmA protein	139
	5.2.9 Storage of recombinant surface display proteins	140
	5.2.10 Storage of <i>L. lactis</i> cells for cell surface display study	140
5.3	Results	141
	5.3.1 The study of binding stability of purified cell wall binding domain of AcmA protein onto the surface of <i>L. lactis</i>	141
	5.3.2 Effect of pH on bound AcmA protein	141
	5.3.3 Influence of pH on cell wall binding of AcmA protein	143
	5.3.4 Effect of LiCl on bound AcmA protein	146
	5.3.5 Pretreatment of <i>L. lactis</i> with TCA	146
	5.3.6 Storage of recombinant cell wall binding proteins	149
	5.3.7 Storage of <i>L. lactis</i> cells for binding study	151
5.4	Discussion	154
VI	CELL SURFACE DISPLAY OF N-TERMINAL REGIONS OF VP1 GENE OF EV71 ON <i>LACTOCOCCUS LACTIS</i> BY USING CELL WALL BINDING DOMAINS OF N-ACETYLMURAMINIDASE AND SERINE PROTEASE	159
6.1	Introduction	159
6.2	Materials and methods	160
	6.2.1 Bacterial strains and plasmids	160
	6.2.2 Truncation of VP1 gene and PCR conditions	161
	6.2.3 Cloning of N-terminal regions of VP1 gene into pSVacm and pSVnp vectors	163
	6.2.4 Verification of recombinant plasmids	165
	6.2.5 Sequencing of recombinant plasmids	165
	6.2.6 Expression of fusion proteins in <i>E. coli</i>	166
	6.2.7 Expression studies by SDS-PAGE and Western blot	166
	6.2.8 Purification of fusion proteins and binding to <i>L. lactis</i>	167



6.2.9	Enzyme-linked immunosorbant Assay (ELISA)	168
6.2.10	Immunofluorescence microscopy	169
6.2.11	Stability assay	170
6.2.12	Binding stability determination of fusion proteins by treatment with 8 M licl	170
6.2.13	Immunogenicity studies	171
6.2.14	Western bolt for the detection of antigen-specific serum antibody	172
6.2.15	Analysis of antigen-specific serum antibody by ELISA	172
6.3	Results	173
6.3.1	Amplification of N-terminal regions of VP1 gene	173
6.3.2	Cloning of N-terminal regions of VP1 gene into pSVacm and pSVnp	175
6.3.3	Verification of recombinant pSVacmVP1 ₁₋₂₀₁ and pSVacmVP1 ₁₀₃₋₃₀₀	175
6.3.4	Verification of Recombinant pSVnpVP1 ₁₋₂₀₁ and pSVnpVP1 ₁₀₃₋₃₀₀	178
6.3.5	DNA sequence analysis of recombinant plasmids	178
6.3.6	Expression of N-terminal regions of VP1 gene as AcmA and NisP fusion proteins	183
6.3.7	Affinity purification of recombinant fusion proteins	186
6.3.8	Study of surface anchoring ability of the fusion proteins	186
6.3.8.1	Whole cell ELISA analysis	186
6.3.8.2	Binding analysis by immunofluorescence	190
6.3.9	Binding stability of fusion proteins on the surface of <i>L. lactis</i>	190
6.3.10	Detection of serum antibody response for AcmA/VP1 _{1-67aa} and AcmA/VP1 _{35-100aa} antigens of VP1 of EV71 in mice	194
6.4	Discussion	206
VII	GENERAL DISCUSSION	210
VIII	CONCLUSION AND FUTURE RECOMMENDATIONS	227
	REFERENCES	230
	APPENDICES	252
	BIODATA OF THE AUTHOR	321



LIST OF TABLES

Table	Page
2.1 Selected examples of surface-displayed proteins on bacteria or yeast	22



LIST OF FIGURES

Figure		Page
2.1	Cell wall of gram-positive bacteria	14
2.2	Cell wall composition of gram-positive bacteria	15
2.3	Diagram of the structures of several anchored surface proteins from various gram-positive bacteria	24
2.4	Diagram of anchoring AcmA protein to cell wall of <i>L. lactis</i>	33
2.5	Diagrammatic representation of three domains in <i>acmA</i> gene of <i>Lactococcus lactis</i> MG1363	34
2.6	Diagrammatic representation of exportation nisin pre-peptide (inactive) into a active (nisin) by removal of its N-terminal leader peptide by surface-located serine protease NisP	37
2.7	Diagrammatic representation of three domains in <i>nisP</i> gene of <i>Lactococcus lactis</i>	38
2.8	Structure of a surface protein linked to the peptidoglycan of <i>S. aureus</i>	43
2.9	Proposed model for the cell wall sorting reaction	44
2.10	Applications of microbial cell surface display	48
3.1	Schematic diagram of the cloning strategy	71
3.2	Agarose gel electrophoresis analysis of PCR amplification of cell wall binding domain of <i>acmA</i> gene by using <i>Pfu</i> DNA polymerase	77
3.3	Agarose gel electrophoresis of cell wall binding domain of <i>nisP</i> gene was amplified using <i>Pfu</i> DNA polymerase	78
3.4	Verification of pSVacm construct by restriction enzymes digestion analysis	81
3.5	Verification of the recombinant plasmid (pSVacm) by PCR	82
3.6	RE analysis of recombinant pSVnp construct	83
3.7	Verification of pSVnp construct by PCR	84



3.8	The sequencing result of pSVacm clone compared with published <i>acmA</i> gene sequence	86
3.9	The sequencing result of pSVnp clone compared with published <i>nisP</i> gene sequence	87
3.10	Amino acid sequence alignment of the C-terminal repeats of AcmA protein (bold), preceding and intervening sequences in C-terminal region of <i>acmA</i> gene	88
3.11	Amino acid sequence alignment of the C-terminal repeat of AcmA protein with C-terminal region of NisP protein	89
3.12	Alignment of deduced amino acid sequences of C-terminal repeat region AcmA of <i>L. lactis</i>	90
3.13	The domain specific homology of C-terminal repeat regions of AcmA protein with LysM (lysin motif) domain of lytic transglycosidase (MltD) of <i>E. coli</i>	92
4.1	SDS-PAGE and Western blot analyses of the over-expressed AcmA recombinant protein	108
4.2	SDS-PAGE and Western blot analyses of the over-expressed NisP recombinant protein	110
4.3	SDS-PAGE analysis of purified recombinant AcmA protein	111
4.4	Whole cell ELISA analysis for AcmA protein binding ability	113
4.5	Whole-cell Ni ²⁺ binding assay for AcmA protein binding ability	115
4.6	Confocal micrographs of AcmA protein binding ability	116
4.7	Confocal micrographs of binding analysis of His-tag pRSET protein	117
4.8	Whole cell ELISA analysis for NisP protein binding ability	119
4.9	Whole-cell Ni ²⁺ binding assay for NisP protein binding ability	121
4.10	Confocal micrographs of NisP protein binding ability	122
4.11	Confocal micrographs of binding analysis of His-tag pRSET protein	123
4.12	The study of binding ability of cell wall binding domain of AcmA to other gram-positive bacteria	125



4.13	The study of binding ability of cell wall binding domain of AcmA to <i>Lactobacillus</i>	126
4.14	The study of binding ability of cell wall binding domain of AcmA to <i>E. coli</i>	128
5.1	ELISA analysis for stability of AcmA protein binding	142
5.2	The effect of pH on bound AcmA protein	144
5.3	The effect of pH on cell wall binding of AcmA protein	145
5.4	The effect of 8 M LiCl on bound AcmA protein	147
5.5	The effect of pretreatment of <i>L. lactis</i> with TCA on binding of AcmA protein	148
5.6	Micrographs of <i>L. lactis</i> cells treated with TCA	150
5.7	The effect of storage on purified recombinant cell wall binding proteins	152
5.8	The storage effect on <i>L. lactis</i> cells for binding study	153
6.1	Diagrammatic representation of truncation of VP1 gene of EV71	162
6.2	Schematic diagram of the cloning of VP1 _{1-201nt} and VP1 _{103-300nt} regions of VP1 gene into pSVacm or pSVnp	164
6.3	Agarose gel electrophoresis analysis of PCR amplification of N-terminal regions of VP1 gene of EV71 by using <i>Pfu</i> DNA polymerase	174
6.4	RE analysis of recombinant pSVacmVP1 ₁₋₂₀₁ construct	176
6.5	RE analysis of recombinant pSVacmVP1 ₁₀₃₋₃₀₀ construct	177
6.6	Verification of recombinant pSVacmVP1 ₁₋₂₀₁ and pSVacmVP1 ₁₀₃₋₃₀₀ constructs by PCR	179
6.7	RE analysis of recombinant pSVnpVP1 ₁₋₂₀₁ construct	180
6.8	RE analysis of recombinant pSVnpVP1 ₁₀₃₋₃₀₀ construct	181
6.9	Verification of recombinant pSVnpVP1 ₁₋₂₀₁ and pSVnpVP1 ₁₀₃₋₃₀₀	



Constructs by PCR	182
6.10 SDS-PAGE and western blot analyses of the over-expressed recombinant fusion proteins (AcmA/VP1 _{1-67aa} and AcmA/VP1 _{35-100aa})	184
6.11 SDS-PAGE and western blot analysis of the over-expressed recombinant fusion proteins (NisP/VP1 _{1-67aa} and NisP/VP1 _{35-100aa})	185
6.12 SDS-PAGE analysis of purified recombinant fusion proteins	187
6.13 Whole cell ELISA analysis for AcmA/VP1 _{1-67aa} and AcmA/VP1 _{35-100aa} protein binding ability	188
6.14 Whole cell ELISA analysis for NisP/VP1 _{1-67aa} and NisP/VP1 _{35-100aa} protein binding ability	189
6.15 Confocal micrographs of the binding of fusion proteins (AcmA/VP1 _{1-67aa} and AcmA/VP1 _{35-100aa}) to <i>L. lactis</i>	191
6.16 Confocal micrographs of the binding of fusion proteins (NisP/VP1 _{1-67aa} and NisP/VP1 _{35-100aa}) to <i>L. lactis</i>	192
6.17 Confocal micrographs of the binding of His-tag pRSET protein	193
6.18 ELISA analysis for stability of binding of fusion proteins (AcmA/VP1 _{1-67aa} and AcmA/VP1 _{35-100aa}) on lactococcal cell surface	195
6.19 Binding strength analysis of fusion proteins (AcmA/VP1 _{1-67aa} and AcmA/VP1 _{35-100aa}) by LiCl treatment	196
6.20 Determination of antibody titers of serum from mice immunized with <i>L. lactis</i> displaying VP1 _{1-67aa} by ELISA	197
6.21 Determination of antibody titers of serum from mice immunized with <i>L. lactis</i> displaying VP1 _{1-67aa} and VP1 _{35-100aa} by ELISA	198
6.22 Determination of antibody titers of serum from mice immunized with <i>L. lactis</i> displaying VP1 _{35-100aa} by ELISA	199
6.23 Determination of antibody titers of serum from control mice immunized with <i>L. lactis</i> (A) and PBS (B) by ELISA	200
6.24 Analysis of serum of immunized mice using EV71 virus coated ELISA plates	202
6.25 Analysis of serum from immunized mice using VP1	



	coated ELISA plates	204
6.26	Detection serum antibody response against VP1 _{1-67aa} or VP1 _{35-100aa} antigens of VP1 of EV71 in mice immunized with <i>L. lactis</i> displaying VP1 _{35-100aa}	205
7.1	Diagrammatic representation of surface anchoring system	221