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

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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

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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

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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, Ni^{2+} 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 form 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 ($VP1_{1\text{-}67aa}$ and $VP1_{35\text{-}100aa}$) 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 $VP1_{1\text{-}67aa}$ and $VP1_{35\text{-}100aa}$ 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 $VP1_{1\text{-}67aa}$ or $VP1_{35\text{-}100aa}$ fusion proteins were able to induce an immune response against the $VP1_{1\text{-}67aa}$ or $VP1_{35\text{-}100aa}$ (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 Biomolecule

Lactococcus adalah sejenis bakteria asid laktik yang telah diguna kan 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 diamplifikasi melalui PCR. Keptu serpihan gen *acmA* dan *nisP* yang telah diamplifikasi 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 $VP1_{1\text{-}67aa}$ dan $VP1_{35\text{-}100aa}$ bagi protein VP1 dari EV71. Bahagian $VP1_{1\text{-}67aa}$ dan $VP1_{35\text{-}100aa}$ 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 $VP1_{1\text{-}67aa}$ dan $VP1_{35\text{-}100aa}$ 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 $VP1_{1\text{-}67aa}$ dan $VP1_{35\text{-}100aa}$ 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 mempunyal 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.

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I certify that an Examination Committee has met on 29th March 2006 to conduct the final examination of Nadimpalli Ravi Sankara Varma on his Doctor of Philosophy thesis entitle "Development of an Anchoring System for Protein Display on the Cell Wall Surface of *Lactococcus lactis* MG1363" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follow:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The Members of the Supervisory Committee are as follow:

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Date: 11 MAY 2006

DECLARATION

I hereby declare that the thesis is based in my original work except for quotations and citation, which have been duly acknowledged. I also declare that it has not been previously or currently submitted for any other degree at UPM or other institutions.

N. Ravi Sankara Varma

NADIMPALLI RAVI SANKARA VARMA

Date: 20-4-2006

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