UNIVERSITI PUTRA MALAYSIA

SPECIES CLASSIFICATION AND MOLECULAR STUDIES OF BILE SALT HYDROLASE (bsh) GENE IN *BIFIDOBACTERIUM* SPP

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By

SHUHAIMI BIN MUSTAFA

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

January 2003

Dedicated to……..

teachers who dedicate the best moments of their life to teach us for a better tomorrow

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

SPECIES CLASSIFICATION AND MOLECULAR STUDIES OF BILE SALT HYDROLASE (*bsh*) GENE IN *BIFIDOBACTERIUM* SPP

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January 2003

Chairperson : Associate Professor Mohd Yazid Manap, Ph.D

Faculty : Food Science and Biotechnology

Molecular methods were used to identify and characterise *Bifidobacterium* isolated from the faeces of breast-fed infants. A *Bifidobacterium* genus-specific primers based on the V9 variable region of the 16S rDNA was used to identify *Bifidobacterium* isolates and to distinguish them from other genera. All the *Bifidobacterium* species tested generated PCR product with the size of approximately 1.35 kb, whereas other genera showed no band with these primers.

Furthermore, a sequence analysis of 16S rRNA gene of *Bifidobacterium* isolates revealed high homology (98% and above) with

Bifidobacterium pseudocatenulatum JCM1200. This result indicates that the 16S rRNA gene sequence analysis is a useful and accurate tool for the identification of the genus *Bifidobacterium*. In addition, a set of *B. pseudocatenulatum* species-specific primer was used as an alternative to identify the species of *B. psedocatenulatum* using PCR technique. It was found that this set of primer was able to generate PCR product with the size of approximately 278 bp in all the *B. pseudocatenulatum* isolates as well as the type strain of *B. pseudocatenulatum* JCM1200. Other species of bifidobacteria gave no band.

A protocol for the rapid fingerprinting technique of *Bifidobacterium* strains and other probiotic microorganisms based on randomly amplified polymorphic DNA (RAPD) has been developed. Three 10-mer primers (GEN 1-60-03, GEN 1-60-06 and GEN 1-60-07) used generated RAPD patterns with DNA fragments ranging from approximately 0.3 kb to 10.0 kb in size. Examination of the DNA fingerprints using cluster analysis showed a significant genetic diversity among the strains tested. Another fingerprinting technique based on the distribution of dispersed repetitive DNA [enterobacterial repetitive intergenic consensus (ERIC) and repetitive extragenic palindromic (REP)] segments in the genome of *Bifidobacterium* and other probiotic bacteria was also examined for the first time using primers derived from the REP and ERIC sequences and

PCR. The patterns of the resulting PCR products were analysed on agarose gel and were found to be highly specific for each species. All the *B. pseudocatenulatum* isolates and *B. pseudocatenulatum* JCM1200 type strain presented an approximately 1.5 kb fragment by ERIC and a 800 bp fragment by REP. These two fragments were not detectable in other species of bifidobacteria. This study demonstrates the presence of ERIC and REP-like elements in the genome of bifidobacteria and other probiotic bacteria.

PCR technique was also used to detect the presence of *bsh* gene in *Bifidobacterium longum* BB536. In this regards, a pair of PCR primers for the rapid detection of *bsh* gene from *B. longum* have been synthesised and have revealed the *bsh* gene of appoximately 970 bp. The *bsh* gene was cloned and sequenced showing a high homology to *bsh* gene previously published. The resulting nucleotide sequence encodes a predicted protein of 317 amino acids with a molecular weight of approximately 35 kDa. The *bsh* gene from *B. longum* was also cloned and expressed in *E. coli* BL21-SI using pRSET-A expression vector. The expressed protein was detected using immunoblot assay.

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

PENGENALPASTIAN SECARA MOLEKULAR SPESIS DAN GEN BILE SALT HYDROLASE (*bsh*) DARI *BIFIDOBACTERIUM* SPP

Oleh

SHUHAIMI BIN MUSTAFA

Januari 2003

Pengerusi : Profesor Madya Mohd Yazid Manap, Ph.D

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Kaedah biologi molekul telah digunakan didalam kajian ini untuk mengenalpasti dan mencirikan isolat bifidobakteria dari najis bayi yang menyusu ibu. Primer genus-spesifik untuk bifidobakteria yang berdasarkan kepada kawasan bolehubah V9 dari jujukan nukleotid 16S rDNA telah digunakan untuk mengenalpasti isolat bifidobakteria dan untuk membezakan mereka dari genera bakteria yang lain. Semua bifidobakteria yang diuji menghasilkan produk PCR bersaiz lebih kurang 1.35 kb, manakala genera yang lain tidak menghasilkan sebarang jalur dengan primer ini.

Kemudian, analisa jujukan gen 16S rRNA dari isolat bifidobakteria menunjukkan persamaan yang tinggi (98% dan keatas)

dengan *Bifidobacterium pseudocatenulatum* JCM 1200. Keputusan ini menunjukkan bahawa analisis jujukan gen 16S rRNA adalah satu kaedah yang berguna dan tepat untuk mengenalpasti genera bifidobakteria. Berikutnya, satu set primer spesis-spesifik telah digunakan sebagai alternatif untuk pengenalpastian *B. psedocatenulatum* menggunakan kaedah PCR. Keputusan yang diperolehi menunjukkan bahawa primer ini berupaya menghasilkan jalur PCR bersaiz kira-kira 278 bp untuk semua isolat *B. pseudocatenulatum* dan *B. pseudocatenulatum* JCM 1200. Spesis lain tidak memberikan sebarang jalur PCR.

Protokol untuk pengecap jarian secara cepat keatas strain bifidobakteria dan mikroorganisma probiotik yang lain telah dilakukan dengan menggunakan kaedah 'randomly amplified polymorphic DNA' (RAPD). Tiga jenis primer 10-mer (GEN 1-60-03, GEN 1-60-06 dan GEN 1-60-007) yang telah digunakan menghasilkan corak RAPD dengan jalur DNA bersaiz dari 0.3 hingga 10.0 kb. Ujian keatas corak cap jari DNA yang diperolehi dengan menggunakan kaedah perkumpulan menunjukkan kepelbagaian genetik yang ketara diantara strain-strain yang dikaji. Kaedah pengecap jarian lain yang berasaskan kepada taburan DNA berulang ['enterobacterial repetitive intergenic consensus' (ERIC) dan 'repetitive extragenic palindromic' (REP)] didalam genom

bifidobakteria dan bakteria probiotik yang lain telah dikaji untuk kali pertama menggunakan primer yang diterbitkan dari jujukan REP dan ERIC. Corak produk PCR yang dihasilkan diatas gel agaros adalah spesifik untuk setiap spesis. Semua isolat *B. pseudocatenulatum* dan *B. pseudocatenulatum* JCM 1200 yang dikaji menghasilkan satu jalur bersaiz 1.5 kb dengan kaedah ERIC dan satu jalur 800 bp dengan kaedah REP. Kedua-dua jalur ini tidak terdapat pada lain-lain spesis bifidobakteria yang dikaji. Kajian ini menunjukkan kehadiran unsur-unsur ERIC dan REP didalam genom bifidobakteria dan bakteria probiotik yang lain.

Kaedah PCR juga telah digunakan untuk mengesan kehadiran gen *bsh* didalam *Bifidobacterium longum* BB536. Untuk tujuan ini, sepasang primer PCR untuk pengesanan secara cepat gen *bsh* telah dihasilkan dan dapat mengesan gen *bsh* bersaiz 970 bp. Gen *bsh* ini telah diklonkan dan jujukan nukleotid yang diperolehi menunjukkan persamaan yang tinggi dengan jujukan nukleotid gen *bsh* yang telah diterbitkan. Jujukan nukleotid yang dihasilkan mengkodkan protin yang mengandungi 317 asid amino yang mempunyai berat molekul kira-kira 35 kDa. Gen *bsh* dari *B. longum* ini juga telah diklonkan dan dizahirkan didalam *E. coli* BL21-SI menggunakan vector pRSET-A. Protin yang dihasilkan telah dibuktikan melalui kaedah immunoblot.

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ATCC	American Type Culture Collection
Bp	base pair
BSH	bile salt hydrolase
$CaCl_2$	calcium chloride
CBAH	conjugated bile acid hydrolase
cfu	colony forming unit
CO_2	carbon dioxide
DCA	deoxycholic acid
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
ERIC	enterobacterial repetitive intergenic consensus
g	gram
h	hour
HCl	hydrochloric acid
IgA	immunoglobulin A
IPTG	isopropyl-β-D-thiogalactopyranoside
IRU	intergenic repetitive unit
JCM	Japan collection of microorganisms
K	kalium
kb	kilobase
kDa	kilodalton
L	litre
LAB	lactic acid bacteria
Lb	luria bertani
mM	millimolar

M	molarity
$MgCl_2$	magnesium chloride
Mg_2SO_4	magnesium sulphate
min	minute
ml	millilitre
Na	natrium
NaCl	natrium chloride
Ng	nanogram
NTSYS	numerical taxonomy system
nm	nanometre
N	normality
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis
PU	palindromic unit
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RAPD	random amplification of polymorphic DNA
rDNA	ribosomal DNA
RE	restriction enzyme
REP	repetitive extragenic palindromic
Rep	repetitive
rRNA	ribosomal RNA
s	second
SAHN	sequential, agglomerative, hierarchical, nested
SDS	sodium dodecyl sulphate
Subsp	subspecies
spp	species
TDCA	taurochenodeoxycholic acid