CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and chemicals

Human lactoferrin cDNA was kindly given from Dr. Deal-Yeul Yu, Head of Animal Molecular Physiology Research unit, Korea Research, Institute of Bioscience and Technology, Kribb Taejon, Korea, The Plasmid DNA was subcloned in *EcoR*I site of pBluscript SK(-) vector (pSKLF). The descriptions of plasmids and bacterial strains used in this study are summarized in Table 2.1. The simple chemicals and enzymes are shown in Appendix.

Table 2.1 Bacterial strains and plasmids used in this study

Bacteria strain or Plasmid	Description[a]	Source
E.coli HB101	RecA, Str^r, Gen^s	Maniatis *et al.*, 1982
B. uniformis BU1001	Tc^r, Em^s, Gen^r, Rif^r	Shoemaker *et al.*, 1986
Plasmid		
R751	IncPβ, Tp^r, Tra^+	Shoemaker *et al.*, 1986
pVAL-1	Tc^r, Ap^r, Cm^r, Em^r, Mob^+, Rep^+	Valentine *et al.*, 1988
pSKLF	2.2-kb fragment containing *hLF* cDNA cloned in *Eco*RI site in pBluescript II SK(-)	Yu D Y
pVLFK	2.2-kb *Bam*HI-*Xho*I fragment of *hLF* inserted in *Bam*HI-*Sal*I site of pVAL-1	This study
pVLFNp	2.5-kb fragment of *nanH* promoter fusion *hLF* inserted in *Bam*HI-*Sal*I site of pVAL-1	This study

a) Abbreviation used for antibiotic resistances (r) or sensitive (s): Ap, ampicillin; Cm, chloramphenical; Em, erythromycin; Gen, gentamycin; Rif, rifampin; Str, streptomycin; Tc, tetracyclin; Tp, trimetroprim. Concentration used to select for antibiotic resistances are given in Materials and Methods. Other abbreviations used: Mob, ability to be mobilized by conjugative element; Rep, ability to replicate in *Bacteroides spp.*; Tra, ability to self transfer; Inc, plasmid incompatibility group; Rec, *E. coli* recombination mutation. Em^r is only expressed in *Bacteroides* strains, and the conjugal *Bacteroides* Tc^r gene is not known to be expressed in *E. coli.*

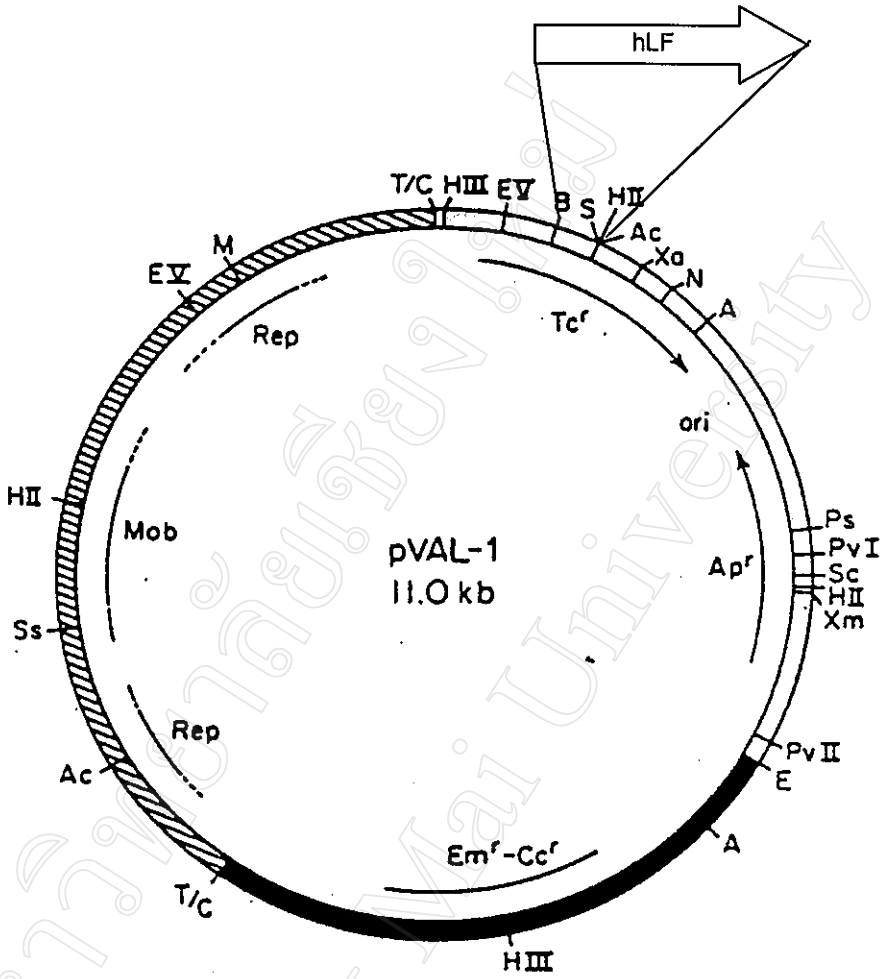

Figure 2.1 The construction of pVAL-1 and subcloning site; Abbreviation used for antibiotic resistance (r) or sensitive (s): Ap, ampicillin; Cm, chloramphenical; Em, erythromycin; Gen, gentamycin; Rif, rifampin; Str, streptomycin; Tc, tetracyclin; Tp, trimetroprim. Concentration used to select for antibiotic resistances are given in Materials and Methods. Other abbreviations used: Mob, ability to be mobilized by conjugattive element; Rep, ability to replicate in *Bacteroides spp.*; Tra, ability to self transfer; Inc, plasmid incompatibility group; Rec, *E. coli* recombination mutation. Em^r is only expressed in *Bacteroides* strains, and the conjugal *Bacteroides* Tc^r gene is not known to be expressed in *E. coli.*

2.2 Construction of *hLF* containing plasmid for expression in *B. uniformis*

2.2.1 Digestion of *hLF* gene from pSKLF

The construction of pVAL-1 is shown in Figure 2.1. Human lactoferrin gene in pSKLF was excised by *Bam*HI and *Xho*I (as shown in Figure 2.2) and plasmid pVAL-1 was excised by *Bam*HI and *Sal*I at 37 °C for 6 hours. The reactions then were applied to 0.7% agarose gel electrophoresis containing 10 mg/ml ethidium bromide. The digested fragments were purified from electrophoresis gel by Quiagen® gel purification Kit. Specific fragment was ligated to *BamHI-SalI* digested tetracycline resistant gene (Tc^r) site of pVAL-1 at 16 °C for 6 hours, generated pVLFK

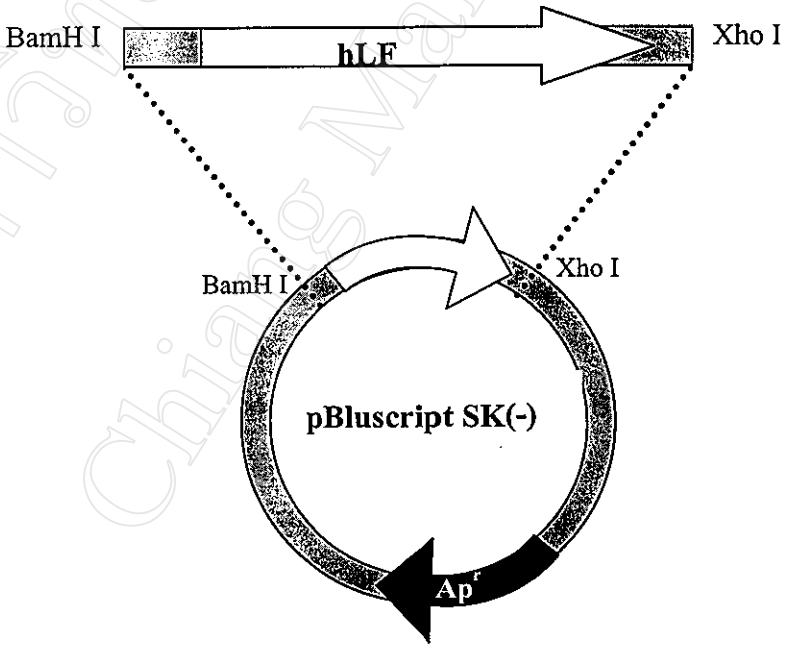

Figure 2.2 Construction and restriction site for subcloning digestion

2.2.2 The *nanH* promoter fusion human lactoferrin gene by PCR

To fuse human lactoferin gene and *nanH* (*Bacteroides* neuraminidase) promotor, specific PCR technique was used. Firstly, human lactoferrin gene in pSKLF and *nanH* promoter were amplified by specific forward and reverse primers showed below. The scheme of PCR principle is shown in Figure 2.3. Primers and conditions used for PCR reaction are as follows;

LFF 5'-ggccgtcgtcgtcgtagtgttcagtggtgcgccgt-3'

LFRSal 5'-tgttggtttgtcgacttacttcctgaggaattcacagg-3'

BamNanHF 5'-ttggtgggatccagataacatccctctctttg-3'

NanHR 5'-acgacgacgacggcctgctgcttgaactaccggtatagcac-3'

1st PCR

Product1

10x Buffer	10 µl
dNTP mix (2.5 mM each)	10 µl
Template YHC46 (500 ng/ml)	10 µl
Primer 10 pmol/µl BamNanHF	5 µl
10 pmole/µl NanHR	5 µl
Ampli Taq gold® DNA polymerase	1 µl
H_2O	59 µl
Total	100 µl

Product 2

10x Buffer	10 µl
dNTP mix (2.5 mM each)	10 µl
Template pSKLF (773 ng/µl)	2 µl
Primer 10 pmol/µl LFF	5 µl
10 pmole/µl LFSal	5 µl
Ampli Taq gold® DNA polymerase	1 µl
H_2O	67 µl
Total	100 µl

PCR condition

96 °C	9 min	
96 °C	1 min	40 cycles
55 °C	1 min	
72 °C	1 min	
72 °C	5 min	
4 °C	∞	

Each PCR product was purified by Quiagen purification kit and subjected to 0.7% agarose gel electrophoresis for purification checking. Gel photo was taken under UV lamp. Purified PCR products 1 and 2 were used for second PCR as templates.

2nd PCR

10x Buffer		10 µl
dNTP mix (2.5 mM each)		10 µl
50 mM $MgSO_4$		2 µl
Template	Product 1 (112 ng/ml)	5 µl
	Product 2 (360 ng/ml)	2 µl
Primer	10 pmol/µl BamNanHF	5 µl
	10 pmole/µl LFRSal	5 µl
Platinum *Pfx*® DNA polymerase		1 µl
H_2O		µl
	Total	100 µl

PCR condition

94 °C		2 min	
	94 °C	15 sec	40 cycles
	55 °C	30 sec	
	68 °C	3 min	
68 °C		5 min	
4 °C		∞	

The PCR products were subjected to 0.7% agarose gel electrophoresis and the size of PCR products were determined against molecular weight marker, λ-*Hind*III (Promega). The *nanH* promoter fusion hLF then was purified from gel by Quiagen® gel purification kit following digested by *Bam*HI and *Sal*I at 37 °C for overnight. Digested fragment then was ligated to *Bam*HI/*Sal*I digested pVAL-1 from 2.2.1 at 16 °C for 6 hours, generated pVLFNp.

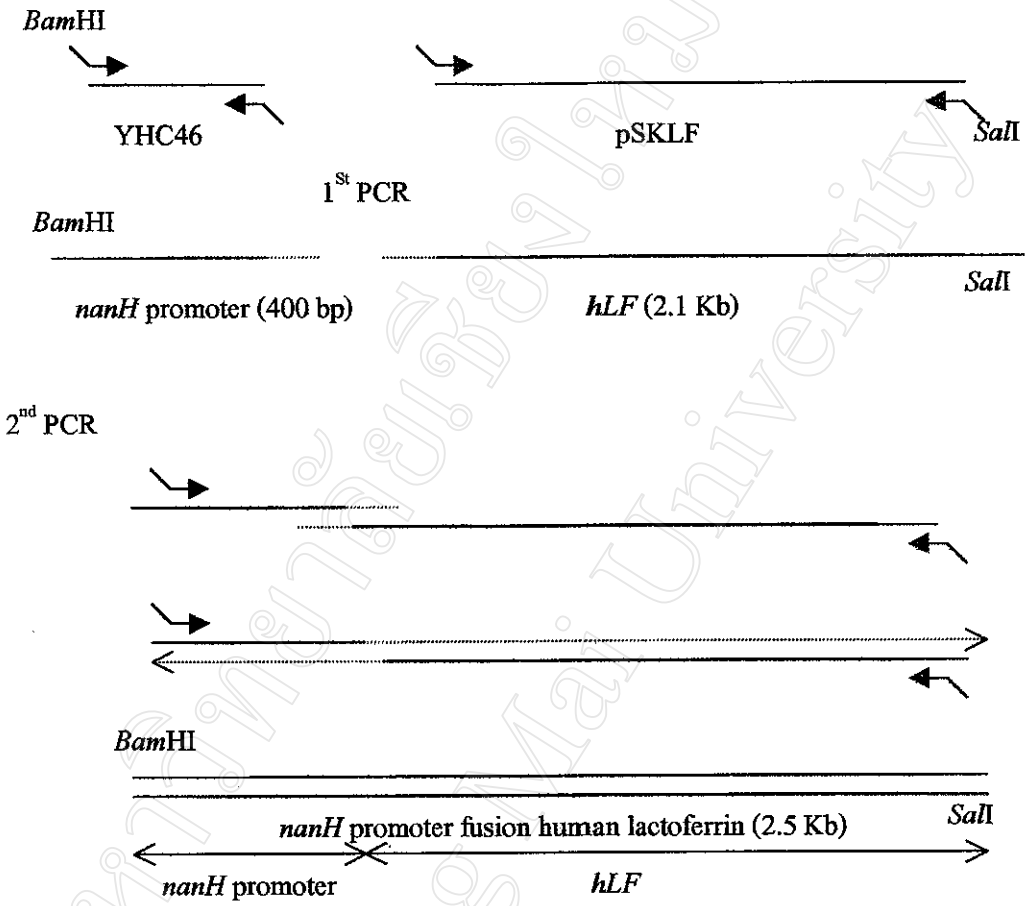

Figure 2.3 Schematic of PCR for fusion *nanH* promoter to human lactoferrin gene

2.2.3 Transformation to *E. coli* HB101

The ligation reaction in 2.2.1 and 2.2.2 and pVAL-1 (1 ng each) were each mixed with 200 µl of *E. coli* HB101 competent cell in small test tube. Mixtures were mixed well and kept on ice for 30 min then heated to 42 °C for 2 min following chilled on ice for 1 min. The 0.8 ml of warmed LB media was added to each tube then incubated at 37 °C for 90 min. One-tenth ml of bacteria culture was divided and spread on LB agar plate containing 50 µg/ml ampicillin. Plates were incubated at 37°C overnight. Ampicillin resistant colonies were isolated to single colony for determining the plasmid harboring. The scheme for ligation and transformation is shown in Figure 2.4.

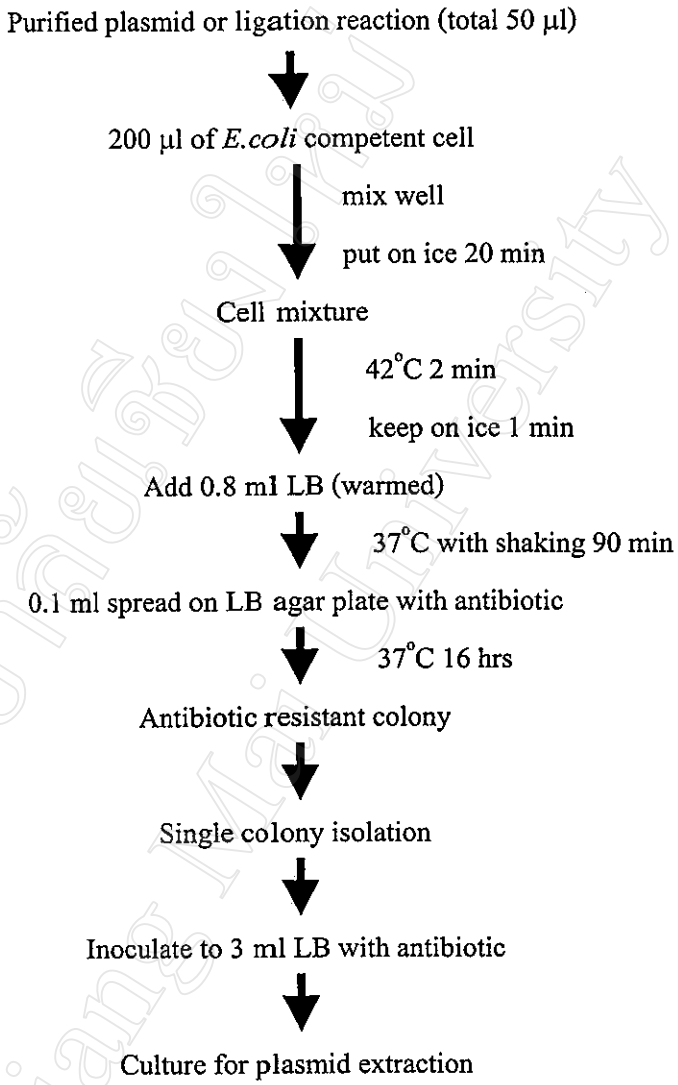

Figure 2.4 Schematic protocol for transformation to *E. coli*

2.2.4 Plasmid extraction

Single colony of each strain was picked up and inoculated to 3 ml of LB medium containing 30 μg/ml amplicillin. Cell was grown at 37 °C with shaking for 14 hours to the stationary phase. One ml of culture was removed and transferred to micocentrifuge tube for collecting cell. After centrifugation, culture supernatant was discarded and 100 μl solution (I) was added and mixed well. The cell suspension was incubated at 37 °C for 20 min then 200 μl of solution (II) was added. The tube was reverting mixed by hand until the solution became clear and viscous. To precipitate DNA, protein and SDS, 150 μl of cold solution (III) was added and mixed by shaking. Mixture was incubated in ice for 10-15 min then centrifuged for 5 min at 4 °C. The supernatant (~400 μl) was poured off into a fresh 1.5 ml microfuge tube and 1 ml 95% ethanol was added and mixed well. The pellet of plasmid was collected by centrifugation about 15 min. Ethanol was carefully poured off and pellet was washed with 1.0 ml 80% ethanol and dried in speed vacuum concentrator. Pellet was resuspended in 50-100 μl water or TE (volume dependent on copy number of plasmid). The schematic for rapid plasmid DNA extraction is shown in Figure 2.5.

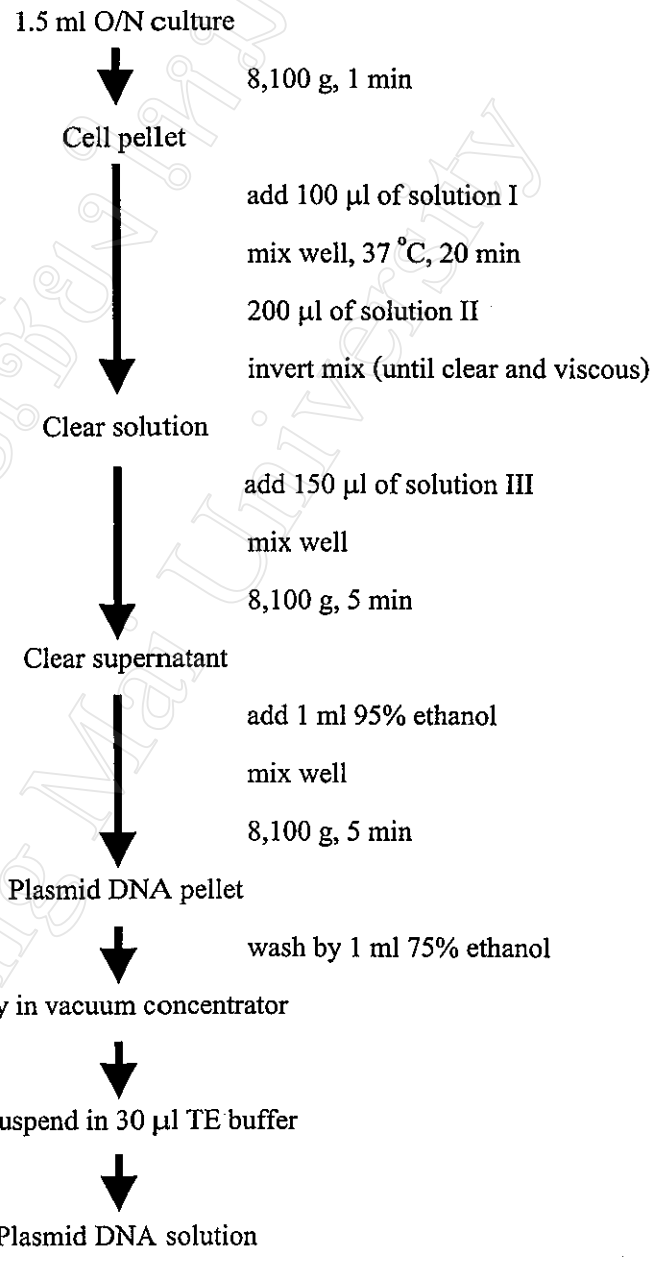

Figure 2.5 Schematic protocol for rapid plasmid DNA extraction

2.2.5 Plasmid determination and upstream sequencing

Extracted plasmid was subjected to 0.7% agarose gel electrophoresis with molecular weight marker, λ-Hind III (Promega). The photos were taken by Kodak digital camera under ultraviolet light. Plasmids were purified from electrophoresis gel by Quiagen® gel purification kit. Purified plasmids were subjected to PCR for sequencing to determine the subcloning site and promoter region by using PVSEQ (5'-aggacagtcgacgtatttct-3') as a sequencing primer. The PCR reaction is as follow;

Template:	pVLFK or pVLFNp	2 µl
Primer:	PVSEQ	4 µl
Reaction mixture		8 µl
Water		6 µl
Total		20 µl

PCR condition

96 °C	1 min	
96 °C	10 sec	25 cycle
50 °C	5 sec	
60 °C	4 min	
4 °C	∞	

After PCR for sequencing, reaction mixture was purified by Centri-Spin column following the procedure of column leaflet. Briefly, hydrated gel column (30 min before use) was centrifuged at 750g for 2 min to remove water from gel. Twenty microliters of sample was transferred to the top of gel then centrifuged at 750 g for 2 min. The purified sample collected in the bottom of the collection tube was dried in speed vacuum concentrator then resuspended in 25 µl of loading buffer. Sample suspension was heated at 95°C for 2 min then subjected to automated DNA sequence analyzer. Then the nucleotide sequences of pVLFK was analyzed for N-lobe by using four designed primers, PVSEQ, LFNF, LFND and LFCD, respectively. The sequencing primers used and annealing sites are shown in Figure 2.6.

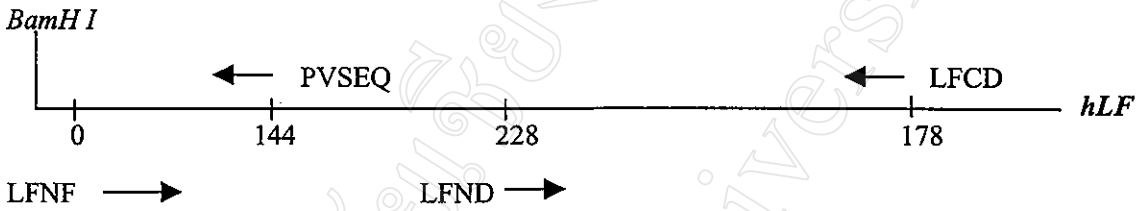

Figure 2.6 The positions of specific primers annealing sites for 5'-terminal sequencing

The sequences of primers used are as follow:

LFNF 5'-tttggccatgggccgtaggagaaggagtgtt-3'

LFND 5'-tgtgttccatggccattgcggaaaacagggccgat-3'

LFCD 5'-tgttgtctcgagcccgggccagatggcagtctttg-3'

PVSEQ5'-aggacagtcgacgtatttct-3'

2.3 Transferring *hLF* gene to *B. uniformis* by *E. coli* HB101 (R751)

2.3.1 Transformation of plasmid and recombinant plasmid to *E. coli* HB101 (R751)

Two hundred microliters of *E. coli* HB101 competent cells containing R751 was mixed with 1 mg of pVAL-1, pVLFK or pVLFNp. Mixtures were mixed well and kept on ice for 30 min then heated to 42 °C for 2 min following chilled on ice for 1 min. The 0.8 ml of warmed LB media was added to each tube then incubated at 37 °C for 90 min. One-tenth ml of bacteria culture was divided and spread on LB agar plate containing 10 μg/ml ampicillin (for pVAL-1 selection) and 10 μg/ml trimetroprim (for R751 selection). Plates were incubated at 37°C overnight. Ampicillin and trimetroprim resistant colonies were isolated as transformant.

2.3.2 Mobilization of *hLF* to *B. uniformis* by *E. coli* HB101 (R751)

B. uniformis strain BU1001 were grown in GAM broth in anaerobic condition for 14 hours then following inoculated to new GAM broth and incubated at 37 °C in anaerobic condition about 4 hour (KU660 about 20) to use as recipients. *E. coli* containing pVAL-1, pVLFK or pVLFNp were grown in LB containing 10 μg/ml ampicillin and10 mg/ml trimetroprim at 37°C with shaking for 14 hours. Overnight culture was inoculated to fresh LB with 10 μg/ml ampicillin and10 μg/ml trimetroprim about 4 hours (KU660 about 25) to use as donors.

The donors were mixed to recipients in the donors-to- recipients ratio 1:1 and 5:1. The two strains mixture were mixed well and centrifuged at 8,100 g for 1 min. Mixed cell pellet was resuspended in 0.1 ml fresh GAM broth. Cell suspension was transferred to mating filter placed on GAM agar plate. Plates were aerobically incubated at 37°C for 16 hours. Mating mixture on the filter was suspended in 3-ml fresh GAM broth. One hundred microliters of suspension was plated on GAM agar plate containing 10 μg/ml erythromycin to select *B. uniformis* harboring pVAL-1 or recombinant plasmid and 200 μg/ml gentamycin sulfate to effect against *E. coli* strain. Plates were anaerobically incubated at 37°C for 48 hours. Single colony of each strain was isolated and kept for further study in 15% glycerol at –70°C.

2.4 Determination of *hLF* existence in *B. uniformis* strain BU1001 (pVLFK and pVLFNp)

2.4.1 Plasmid extraction from *B. uniformis*

B. uniformis strain BU1001 (pVAL-1, pVLFK and pVLFNp) were grown in GAM broth with 10 μg/ml erythromycin in anaerobic condition for 14 hours. One milliliter of culture was transferred to a microcentrifuge tube. The plasmid extraction procedure was same as the method in 2.2.4. Plasmid DNA was identified by running on 0.7% agarose gel electrophoresis compared to λ-*Hind*III molecular markers.

2.4.2 PCR for detection of *hLF* gene in *B. uniformis*

One milligram of plasmid DNA was transformed to *E. coli* HB101 as described in 2.3.1. Plasmids were extracted from *E. coli* harboring each plasmid by method in 2.2.4 and purified by Quiagen® purification kit. Purified plasmids were subjected to PCR to amplify lactoferrin gene using primers as follows;

LFNF 5'-tttggccatgggccgtaggagaaggagtgtt-3'

LFC 5'-gggttctcgagttacttcctg aggaattcacaggc-3'

PCR reaction

10 X Buffer	10 μl
dNTP mix (2.5 mM each)	10 μl
Template extracted plasmid 500 ng/μl	
pVAL-1, pVLFK and pVLFNp	2 μl
Primer 10 pmol/μl LFN	5 μl
10 pmole/μl LFC	5 μl
Ampli Taq gold® DNA polymerase	1 μl
H_2O	67 μl
Total	100 μl

PCR condition

96 °C	9 min	
96 °C	1 min	40 cycles
55 °C	1 min	
72 °C	3 min	
72 °C	5 min	
4 °C	∞	

PCR products were subjected to 0.7% agarose gel electrophoresis. Gel photo was taken under UV lamp.

2.5 Detection of gene expression by Northern hybridization

2.5.1 RNA extraction

B. uniformis strain BU1001 (pVAL-1), BU1001 (pVLFK) and BU1001 (pVLFNp) were grown in GAM broth with 10 µg/ml erythromycin in anaerobic condition for 6 hours until KU reached to 40-60. Ten milliliters of culture was transferred to a sterilized centrifuge tube. Cells were collected by centrifugation at 12,000 g for 10 min. Cell pellet was suspended in 0.5 ml lysis buffer and then transferred to a 1.5 ml microfuge tube. Bacteria cells were frozen on dry ice-ethanol, then thawed and lysed by sonication (30W) 3 times for 10 second each. The lysate was incubated at 37 °C for 60 min. RNA was extracted by adding equal volume of PCI (phenol-chloroform-isoamyalcohol), then vortexed and centrifuged at 12,000 rpm for 5 min. The upper aqueous phase was removed and transferred to a new tube. Then an equal volume of chloroform: IAA was added into the tube. The mixture was mixed well and centrifuged at 8,100 g, for 5 min. The aqueous phase was collected and 15 µl of 5M sodium chloride was added. To precipitate total RNA, 1 ml of 95% cold ethanol was added to the collected aqueous phase. RNA precipitant was kept in –20 °C for 1-2 hours for complete precipitation. RNA was collected by centrifugation at 8,100 g, 4°C for 15 min. Ethanol was discarded and RNA pellet was rinsed by cold 70% ethanol. RNA pellet was dried by air.

2.5.2 DNase treatment of extracted RNA

Total RNA from 2.5.1 was dissolved in DNase digesting buffer and 4 µl of 2.5 mg/ml RNase free DNase I. Reaction mixture was incubated at 37°C for 60 min. DNase-treated RNA was isolated by phenol extraction as described in 2.5.1. RNA was precipitated by adding 10 µl of 5M sodium chloride and 600 µl of cold ethanol and kept in –20°C overnight to complete the precipitation. RNA precipitate was collected by centrifugation at 8,100 g, 4 °C for 15 min, washed by cold 75% ethanol, and then dried by air. RNA was dissolved in 100 µl of DEPC-treated water and RNA concentration was determined by measuring absorbance at 260 and 280 nm.

2.5.3 Sample preparation and agarose gel electrophoresis

The mixture of RNA for elecrophoresis is as follow;

1 µg/µl of total RNA	5.5 µl
10 X MOPS buffer	1.0 µl
Formaldehyde	3.5 µl
Formamide	10 µl
Total	20 µl

The mixture was heated at 65 °C for 15 min and immediately chilled in ice for 2 min. Before loading to electrophoresis, 2 µl of loading buffer was added. RNA samples were electrophoresed in 1% Seakem agarose gel by using voltage at 50V for 90 min. After electrophoresis, gel was immersed in denaturing buffer containing 1 µg/ml ethidium bromide for 20 min and following in neutralized buffer for twice for 20 min. Gel photo was taken under UV lamp with scale marker for 23s and 16s ribosomal RNA for molecular markers

2.5.4 Preparation of digoxigenin-labeled *hLF* cDNA probe

To construct DIG-labeled N-terminal and C-terminal c-DNA probe for detection *hLF* mRNA, *hLF* N-terminal was firstly amplified by using PCR with specific primers.

LFNF 5'-tttggccatgggccgtaggagaaggagtgt-3'

LFCD 5'-tgttgtctcgagcccgggccagatggcagtctttg-3'

C-lobeF 5'-ggtttgcatatggcccggcgtgcgcgggtcgtgt-3'

C-lobeR 5'-gggttctcgagttacttcctgaggaattcacaggc-3'

The reaction is as follow;

PCR reaction

10x Buffer		10 µl
dNTP mix		10 µl
Template pKLF (500 ng/µl)		2 µl
Primer	10 pmol/µl LFNF	5 µl
	10 pmole/µl LFCD	5 µl

or

Primer	10 pmol/µl C-lobeF	5 µl
	10 pmole/µl C-lobeR	5 µl
Ampli Taq gold® DNA polymerase		1 µl
H_2O		67 µl
	Total	100 µl

PCR condition

94 °C	9 min	
94 °C	1 min	40 cycles
50 °C	1 min	
72 °C	1 min	
72 °C	5 min	
4 °C	∞	

PCR products were purified by Quiagen® purification Kit and dissolved in distilled water. Two microgarms of N-terminal or C-terminal DNA was heated at 99.9 °C for 10 min and then chilled on ice. The reaction mixture consisted of 2 µl of denatured DNA, 2µl of hexamer oligonucleotide, 2.5 µl dNTP mixed and 1 µl of Klenow polymerase. Mixture was incubated at 37 °C for 30 min, then 2 µl of EDTA and 2.5 µl of 4M lithium chloride was added and mixed well. To precipitate DIG-labeled cDNA, 75 µl of ethanol was added, following kept in -80°C for 1 hour or more. DNA precipitate was isolated by centrifugation at 12,000 rpm 4°C for 15 min. DNA pellet was washed and dried by air. DNA probe was suspended in 50 µl TE buffer and concentration was measured.

2.5.5 Northern hybridization analysis

Migrated RNA from gel was transferred to Hybond N+® nylon membrane by air dry blotting. The blotting apparatus was set up as shown in Figure 2.7. After 16 hours of blotting, membrane was taken from the blotting apparatus and baked at 80 °C for 2 hours in a hot air oven. Membrane was exposed to ultraviolet light at 1200 KW for 3 times in UV-cross linker to cross-link RNA to membrane. Membrane was pre-hybridized in hybridizing buffer at 55 °C for 30 min following hybridized with digoxigenin (DIG)-labeled N-lobe *hLF* cDNA at 55°C for 2 hours. Washing steps of hybridized membrane were once with 2X SSC/0.1%SDS at room temperature followed by twice of 0.1X SSC/0.1%SDS at 55 °C and once with buffer 1. Membrane was blocked by gelatin for 30 min at room temperature, and then incubated in alkaline phosphatase-conjugated anti-DIG antibody at 25 °C for 30 min. To visualize the Emission band, membrane was sprayed with CSPD as substrate and exposed to Fuji RX-U X-ray film. The size of mRNA band was estimated against 23s and 16s rRNA.

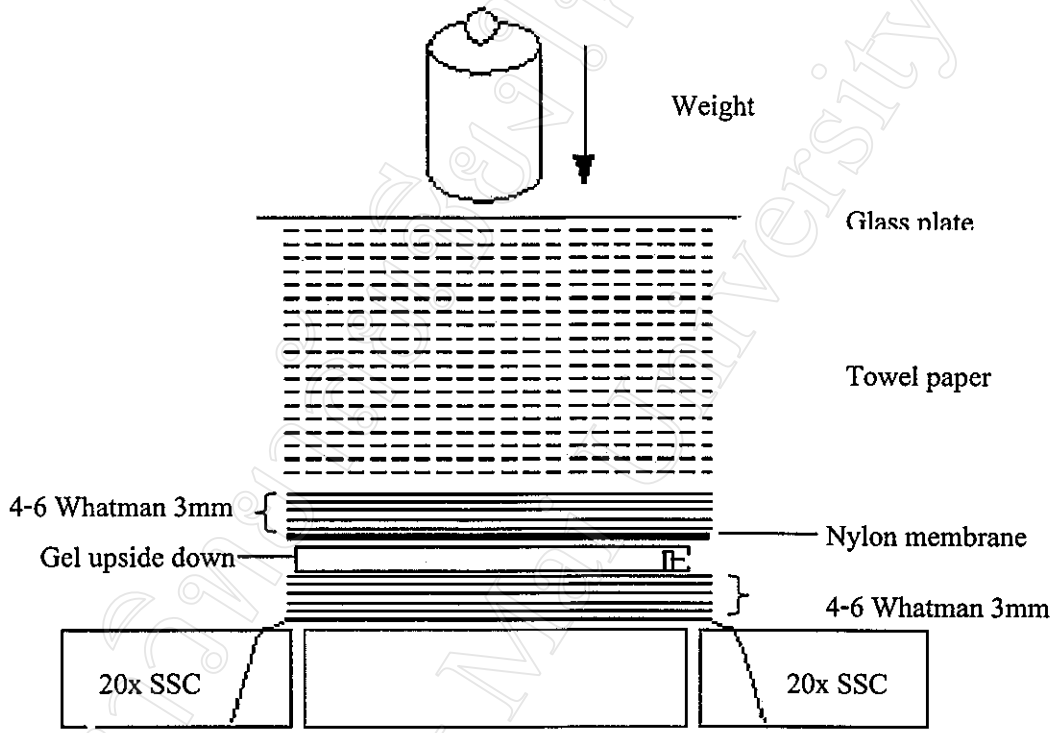

Figure 2.7 Air-dried blot apparatus setting for RNA-membrane transferring

2.6 Detection of hLF gene products by Western blot analysis

2.6.1 Sample preparation and SDS-PAGE

B. uniformis strains BU1001 (pVAL-1), BU1001 (pVLFK) and BU1001 (pVLFNp) were grown in GAM broth with 10 μg/ml erythromycin in anaerobic condition for 6 hours until OD600 reached to 0.4-0.6. Ten milliliters of the culture was transferred to centfuge tube. Cells were collected by centrifugation at 12,000g for 10 min. Cell pellet was washed by 0.15% KCl and centrifuged at 12,000 g for 10 min, weighed and suspended in 3 volume of 0.15% KCl of cell wet weight. Bacterial cells were lysed by sonication on ice for 1 min and stand on ice for 1 min, repeated 5-10 cycles. Cell homogenate was centrifuged at 9000 g at 4 °C for 20 min. Supernatant was collected as cellular protein and protein concentration was measured by the Lowry's method. Two microgram of soluble cellular protein was boiled in loading buffer and electrophoresed on 10% SDS-PAGE at 20 mA for 16 hours.

2.6.2 Preparation of bacterial outer membrane

This method was derived from Kotarski and Salyers (Kotarski and Salyers, 1984) as shows in Figure 2.8. Two hundred milliliters of O/N culture of *B. uniformis* strain BU1001 (pVAL-1) and BU1001 (pVLFK) was centrifuged to collect bacterial cells at 10000 g for 15 min. Cell pellet was washed twice by potassium phosphate buffer (KPB) pH 7.0 and then suspended in 4 ml of KPB containing 50 μg/ml DNase I and 20 μg/ml RNaseA. Bacterial cells were lysed by sonication on ice for 1 min stand on ice for 1 min, repeated 5-10 cycles. Homogenate was kept on ice for 30 min then was centrifuged at 17000 g for 15 min, 4°C. Supernatant was collected and transferred to cleaned ultracentrifuge tube following centrifuged at 200000 g for 2.5 hr, 4°C. Membrane pellet was collected and suspended in 2 ml KPB by homogenizer then centrifuged again at 200000 g for 2.5 hr, 4°C. Supernantant (cytosol) and pellet (outer membrane) were separately collected and cell pellet was suspended in 2 ml KPB by homogenizer. Protein concentration was measured by Lowry's method. Total cell homogenate (S9), cytosolic fraction (S200) and membrane fraction were electrophoresed on 10% SDS-PAGE at 20 mA for 16 hours.

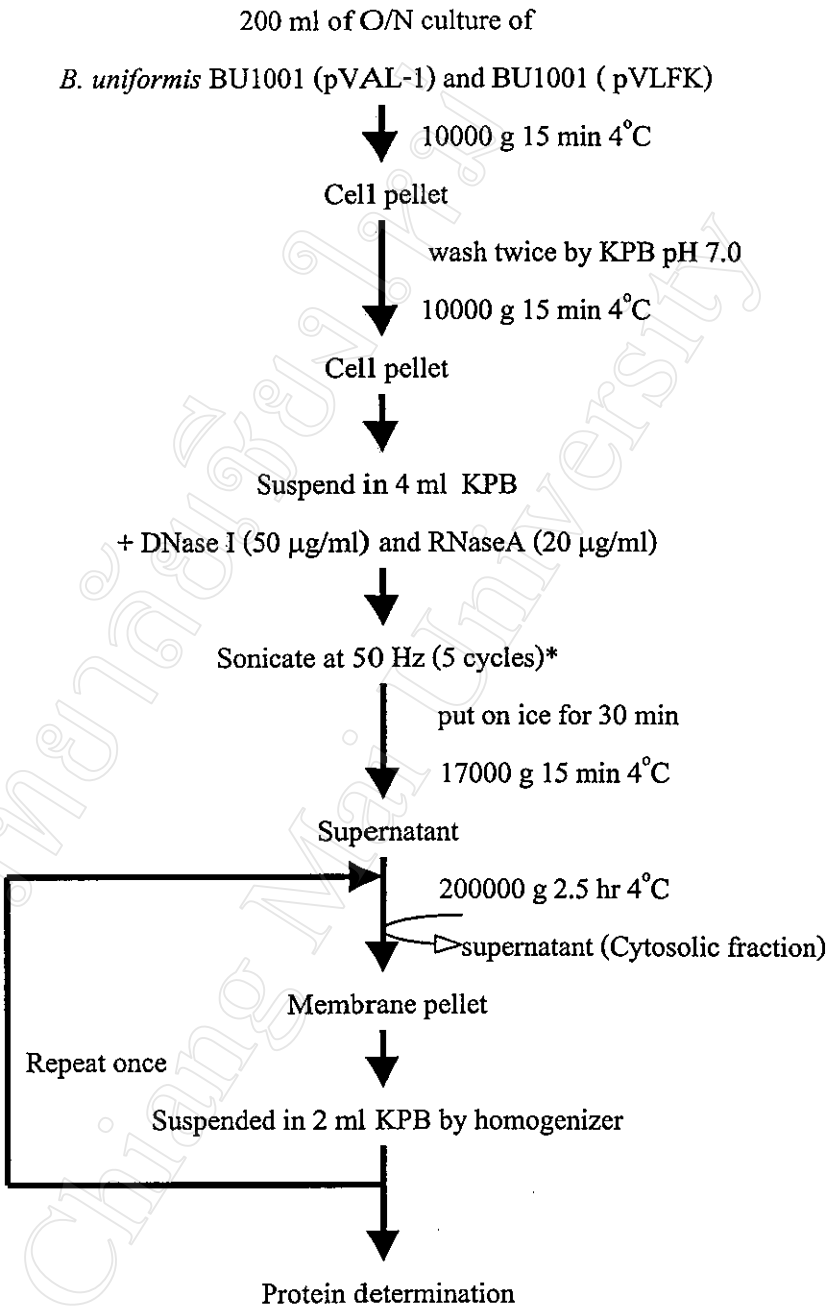

Figure 2.8 Schematic protocol for outer membrane and cytosol preparation

* Repeat sonication 1 min and put keep on ice 1 min for 5 times

2.6.3 Western blot analysis

Migrated proteins in SDS-PAGE gel were blotted onto PVDF membrane by semi-dry blotting at 20 V, 0.5 mA ampere for 60 min. Membrane was washed and blocked with 5% skim milk, then incubated with rabbit anti human lactoferrin antisera at 1:3000 dilution for 2 hours. After washing by TTBS, specific proteins were detected by Biorad Chemo Enhancement Detection Kit. Briefly, membrane was incubated by streptavidine labeled goat anti rabbit IgG at 1:3000 dilution, following biotin peroxidase conjugate. After well washing by TTBS and finally by TTB, bands were visualized by immersed membrane in color developing solution. To confirm the presence of specific proteins, binding of anti-human LF antisera was detected by incubation of membrane with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody for 2 hours. Membrane was sprayed with CSPD as substrate. Visualized system was same as Northern blot analysis.

2.7 Co-culture of *B. uniformis* strain pVAL-1 or pVLFK with *E.coli* HB101

Overnight culture of *B. uniformis* strain BU1001 (pVAL-1) or BU1001 (pVLFK) was inoculated to 9 ml GAM broth without erythromycin and anaerobically incubated in 37°C for 5 hours or until KU 660 reached to 80. *E. coli* HB101 was grown in L-broth until cell number was similar to *B. uniformis*. One milliliter of *E. coli* cells were collected and resuspended in fresh GAM broth. *B. uniformis* each strain and *E. coli* HB101 in GAM broth were mixed together and incubated at 37 °C without shaking. Co-cultured mixture was aspired and diluted by saline, subsequently plated on LB agar plate at time 0, 1, 3 and 6 hours respectively. Plates were incubated aerobically. The cell numbers of *E. coli* were determined in two independent experiment in triplicate.

2.8 Bacterial culture and animal treatment

2.8.1 Growth of *B. uniformis* strains BU1001 (pVAL-1) and BU1001 (pVLFK)

B. uniformis BU1001 harboring pVAL-1 and pVLFK from the stock were grown in GAM broth containing 10 μg /ml erythromycin for 24 hours. The cultures were inoculated to fresh GAM broth containing 10 μg /ml erythromycin then continuously cultured and the number of bacteria in each strain was counted at indicated times.

2.8.2 Animal treatment

Twenty-three-hour culture was given to rats as a drinking water. The numbers of bacteria in the cultures of strains pVAL-1 and pVLFK are 2.2×10^{10} and 2.0×10^{10} colony forming unit (CFU)/ml respectively (at the stationary phase of the growth of both strain). Five weeks old F344 male rats, obtained from Japan Charles River Inc. were given pellet diet, water and experiment drinks *ad libitum* during the experiment (Fig. 2.9). After 1 week of pretreatment all rats received subcutaneous injection of AOM in the dose of 15 mg/kg body weight once a week for two weeks. Rats were weighed weekly and were sacrificed on the third week after the second AOM injection.

2.8.3 Aberrant crypt foci evaluation

After the rats were killed the colon and rectum were removed and examined for ACF as described by Bird *et. al.* (1995). The colons were bound with string at the anal site, then injected with 10% formaldehyde-PBS and tightly bound at the caecum site. The colons were taken out and placed on ice for 15 min. They were then cut open along the longitudinal median axis, washed with 10% formaldehyde-PBS. They were cut into three parts, the rectum (two centimeters from the anus), proximal half and distal half. All parts were placed on a piece of filter paper, flattened and stretched by cover with a second piece of filter paper, then fixed in 10% formaldehyde-PBS. The pieces of colon were stained with 0.2% methylene blue-saline to visualize the crypt. They were placed on microscope slide with the mucosal side up and foci of aberrant crypt were scored under the light microscope at a magnification of 40x. Aberrant crypt focus was distinguished from the surrounding normal crypt by its increased-size as shown in Figure 1.3.

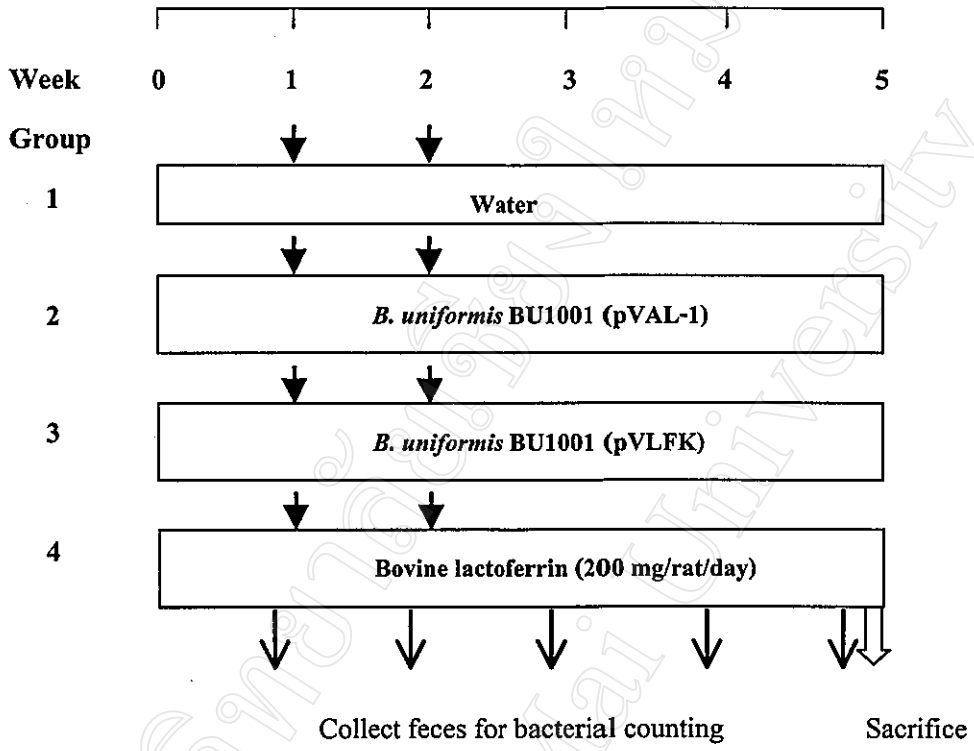

Figure 2.9 Protocol for animal treatment 1, ▼ = AOM 15 mg/ kg body weight

2.8.4 The number of bacteria in rat feces

Fresh rat feces were collected weekly. Weighting and dilution of fecal specimen were carried out under anaerobic and sterile condition. Bacteria suspension was plated on GAM agar plate with and without erythromycin. Plates were incubated in both aerobic and anaerobic condition. Number of fecal bacteria was scored in aerobic, anaerobic and Em^r-anaerobic bacteria. To confirm whether Em^r-anaerobic bacteria were transformants of the *B. uniformis*, some of Em^r-anaearobic bacteria were randomly isolated and identified by the API-20® anaerobic bacteria identification test kit.

2.9 Effects of various species of *Bacteroides* on ACF formation

Male Fishers F344 rats at five weeks old, obtained from Japan Charles River Inc. were given pellet diet, water and experiment drinks *ad libitum* during the experiment. The various species of *Bacteroides* used in this study which were characterized by Paster and colleagues (Pasture *et al.*, 1994) were *B. uniformis* BU1001, *B. uniformis* ATCC8492, *B. thetaiotaomicron* KYU 1, *B. uniformis* KYU 2, *B. fragillis* KYU 3, *B. ovatus* KYU 4, *B. ovatus* ATCC8483, *B. distasonis* ATCC8503, *B. eggethrii* ATCC27754, *B. thetaiotaomicron* Werner E50, *B. vulgatus* ATCC8482, *B. caccae* JMC9498, *B. merdae* JMC9497 and *B. stercoris* JMC9496. Bacterial cultures were given to rats as drinking water in experimental group as shown in Figure 2.10. After 5 weeks, the rats were killed, and the colons and rectums were removed and examined for ACF as described in 2.8.3.

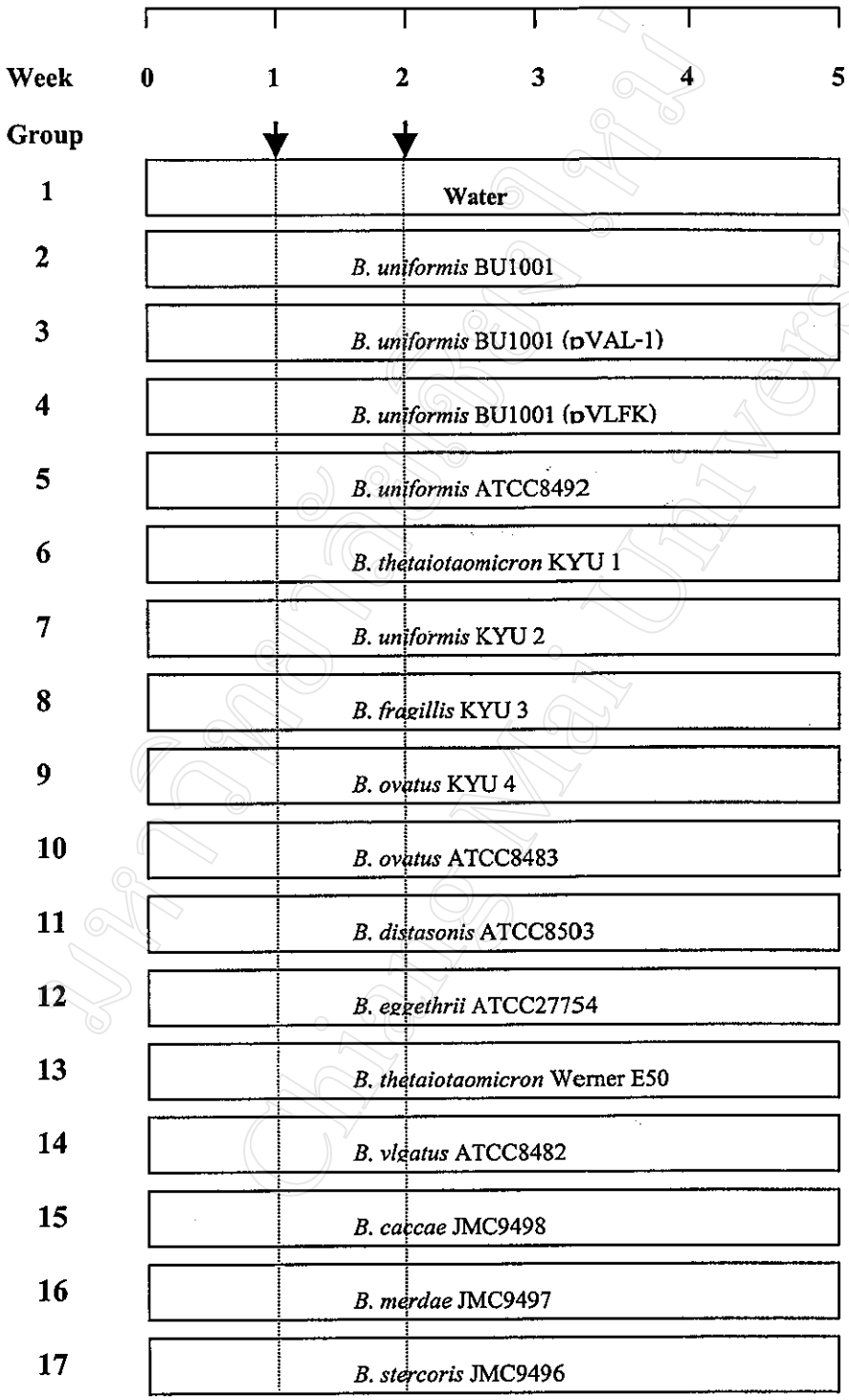

Figure 2.10 Protocol for animal treatment 2, ↓ = AOM 15 mg/kg body weight

2.10 The activity of bacterial β-glucuronidase

2.10.1 β-glucuronidase activity in cell free extracts and culture supernatants of *B. uniformis*

B. uniformis strains BU1001 (pVAL-1) and BU1001 (pVLFK) and *Clostridium perfingen* strain GAI 0668 (positive control) were grown in GAM broth with 10 μg/ml erythromycin in anaerobic condition for 46 hours. Ten milliliters of culture was transferred to centrifuge tube. Cells were separated by centrifugation at 12,000 g for 10 min and the culture supernatant was collected for enzyme assay. Cell pellet was washed by phosphate buffer pH 7.0, centrifuged at 12,000 g for 10 min, weighed and suspended in 3 volume of PB/EDTA of cell wet weight. Bacterial cells were lysed by 5-10 cycles of sonication on ice for 1 min and stand on ice for 1 min. Cell homogenate was centrifuged at 9000 g, 4°C for 20 min. The supernatant was collected as cell free extract and protein concentration was measured by the Lowry's method.

2.10.2 Fecal β-glucuronidase activity

Feces from group 1-4 (Figure 2.10) were collected before second AOM injection, 3 hour and 3days after second AOM injection and kept at –80 °C until assay for enzyme activity. Before assay, each feces were weighed and suspended in 3 volume of sodium phosphate buffer/EDTA followed by centrifugation for 10 min at 3000 g. The suspension was determined for protein concentration by the Lowry's method.

2.10.3 β-glucuronidase assay

Fecal-β-glucuronidase activity was assayed by using p-nitrophenol (PNP)-β-D-glucuronide as a substrate. The reaction mixtures consisted of 20 μl of 100mM PNP-β-D-glucuronide, 40μl of 100 mM phosphate buffer pH 7.0 containing 0.5 mM EDTA and 130 μl water. The enzyme reaction was started by adding 10 μl of fecal sample from each rat. The mixture was incubated in water bath at 37 °C for 30 min and then one ml of 0.2 M glycine-0.2 M NaCl pH10.1 was added to stop the reaction. The PNP liberated was measured by spectrophotometer at 400 nm. Enzyme activity was expressed as μmole of p-nitophenol generated per minute per milligram of protein. The schematic determination of enzyme activity is shown in Figure 2.11.

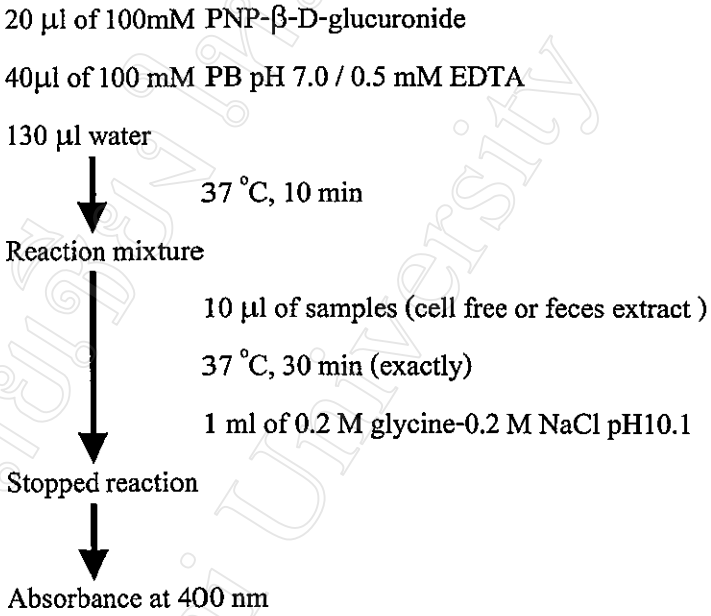

Figure 2.11 Scheme of β-glucuronidase determination

2.11 Effect of lactoferrin-producing *B. uniformis* strain KYU2 on the ACF formation in the colon of AOM-treated rats

Vector plasmid pVAL-1 or *hLF* carrying plasmid pVLFK or was cloned into *B. uniformis* strain KYU2 as described in 2.3. The original *B. uniformis* strain KYU2 and transconjugants *B. uniformis* KYU 2 (pVAL-1) and KYU 2 (pVLFK) were cultured as described in 2.8.1 and then given to rats as drinking water described in 2.8.2. The experiment design is shown in Figure 2.12. Five weeks after AOM treatment rats were sacrificed and the number of aberrant crypt foci was determined as described in 2.8.3.

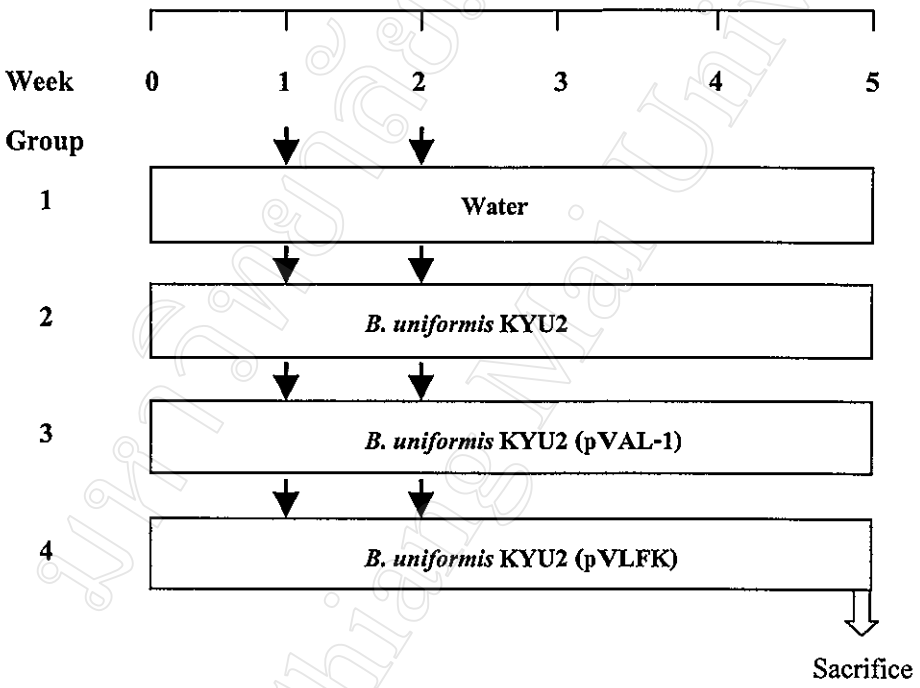

Figure 2.12 Protocol for animal treatment 3, ↓ = AOM 15 mg/ kg body weight

2.12 Statistical analysis

The numbers of ACF, AC/f and number of bacteria were statistically analyzed by χ-square test using Stat view statistics analyzing software for Macintosh version 5.0.