CHAPTER 4

DISCUSSION AND CONCLUSION

Primary prevention of the colon cancer is a useful mode for reducing the mortality rates of this disease (Bertangnolli *et al.,* 1997). One useful strategy for preventing colon cancer is the colonization of beneficial intestinal bacteria in the colon, or intestinal bacteria that acquire new functions by introducing biosynthetic gene(s) for chemopreventive agents. Recently, an increasing interest has been shown in the anticarcinogenicity of lactoferrin in the field of cancer chemoprevention. In this study, a recombinant *B. uniformis* strain producing human lactoferrin was constructed and then evaluated for the preventive effect of its culture on colon carcinogenesis.

4.1 Construction of lactoferrin-producing *B. uniformis*

The human lactoferrin-expressing plasmids were constructed in two different orientations. Initially, pVLFK was simply constructed by inserting a *LF* fragment (*Bam*HI/*Sal*I) directly to pVAL-1 (*Bam*HI/*Xho*I), which included 18 amino acid residues of an hLF-prepeptide. Additionally, the orientation of pVLFNp construction was a *nanH* promoter, Bacteroides signal sequence (22 amino acid residue) and human lactoferrin cDNA, consecutively (Figure 3.3). This construction of hLF-expressing plasmid, pVLFNp, was expected to secrete a high quantity of recombinant lactoferrin by the function of a *Bacteroides* signal peptide (secreted from a bacterium host) and *nanH* promoter (increased transcriptional level). The *nanH* was the gene that expressed the neuraminidase enzyme during the growth of *B. fragilis* (Fraser and Brown, 1981 and Godoy *et al.,*1993). This gene was expressed under the driven promoter of *nanH* located on the downstream of the *nanH* gene (Ono *et al.,*1994). Therefore, the genomic DNA of *B. fragilis* YHC46 was used as a template to amplify the *nanH* promoter for protein fusion. After this DNA fragment was sequenced, it showed the complete bacterial transcription system that included –10 and -35 regions and a ribosomal binding site. Tweedie *et al.* (1994) had constructed *hLF-*

expressing plasmid in BHK cell. They tried to express either a half N-lobe- or C-lobe-expressing plasmid. For C-lobe construction, alanine was replaced by glycine, and then the N-terminal pre-peptide was placed together with C-lobe cDNA (Tweedie, *et al.*, 1994). This indicated that a pre-peptide might be necessary for the expression of recombinant lactoferrin. Takimura and colleague (1997) indicated the necessity of a signal peptide on the secretion of recombinant human interleukin-2 from *Bacillus bravis* in the culture medium (Takimura *et al.*, 1997). They also modified the appropriate sequence of signal peptides to increase the secretion efficiency of a recombinant protein. The construction of pVLFK showed the presence of lactoferrin pre-peptide, while pVLFNp contained the signal peptide of Bacteroides in order to secrete the recombinant peptide into culture media. The production of rhLF in both orientations of plasmids then followed after investigation.

Because of the oxygen sensitivity of anaerobic bacteria, especially *Bacteroides spp.*, it was difficult to prepare a competent cell for heat shock transformation in order to transfer the plasmid to bacterial cells. The *E.coli-Bacteroides* shuttle vector, pVAL-1, was then constructed by the combination of two plasmids, pB8-51 and pTB1, in which the mobilization region that is recognized by R751 was fully functional (Valentine *et al.*, 1988). The broad host range IncP beta plasmid R751 could mobilize itself and the chimeric plasmid from *E. coli* to *Bacteroides spp.*, but they could not be maintained in *Bacteroides spp*. This transconjugal system was used to introduce the chimeric plasmid to Bacteroides. In this study, pVAL-1 was used to construct plasmids for expressing the *hLF* gene in *B. uniformis*. pVAL-1 was mobilized by R751 from *E. coli* to *E. coli* at a frequency of 10^{-3} (Valentine *et al.*, 1988). The mobilization frequency of pVAL-1 was therefore comparable to that of the construction of Shoesmaker and colleague (1986), in which the mobilization region of pB8-51 was fully functional (Shoesmaker *et al.*, 1986). Unlike those constructions, pVAL-1 can replicate in *Bacteroides spp.* They showed the mobilization of pVAL-1 from *E. coli* to *Bacteroides spp.* at a frequency of 10^{-4}. Valentine *et al.* (1988) determined the stability of pVAL-1 in *E. coli* and *B. uniformis*. They suggested that pVAL-1 was more stable in *E. coli* than in Bacteroides. When *E. coli* containing pVAL-1 was grown without drug selection, there was less than 0.3% loss of TC^r after approximately 10 and 20 generations. This shuttle vector was not as stable in *B. uniformis*, which showed after approximately 10 generations of growth without Em selection, and only 60% of the *B. uniformis* colonies were Em-

resistant. At the end of three consecutive transfers (~20 generations) only 2 to 4% of the *B. uniformis* colonies were Em-resistant. They found that none of 6 colonies of Em^s *B. uniformis*, which were screened, contained any plasmid DNA, and suggested that the a loss of Em^r was probably due to loss of plasmid and not simply to a loss of region that carries the Em^r gene. However, it might be a suitable plasmid for carrying the *hLF* gene to *B. uniformis*. The transconjugation frequency in this study was in the order of 10^{-4}, which was similar to that reported by Valentine and colleague (1988) (Valentine *et al.,* 1988). However Thomson and Flint successfully transformed shuttle plasmid pDP1 (19 kb) in *B. uniformis*. The frequency of transformation was greater than 10^6 erythromycin resistant transformants per microgram DNA (Thomsom and Flint, 1989). At that point, Smith *et al.* (1990) deduced the optimal electroporation parameters to transform a variety of plasmid DNAs to *Bacteroides spp.* (Smith *et al.*, 1990). Whitehead and Hespell (1990) successfully cloned a xylanase gene from ruminal bacterium, *B. ruminicola* 23 to *B. fragilis* and *B. uniformis* by using pVAL-1 (Whitehead and Hespell, 1990). The cloned gene was expressed in both species. That was the first demonstration of colonic *Bacteroides spp.* expressing a gene from aruminal *Bacteroides spp.*. Then Gardner *et al.* (1996) constructed a carboxymethyl cellulase (CMCase) gene under xylanase promoter and expressed *B. uniformis*. The xylanase promoter allowed the *B. uniformis* transconjugant to produce large amounts of the reconstructed CMCase, which presented on the outside of the cell. In this study the eukaryotic gene was initially expressed in *B. uniformis* and used as the promoter of *nanH* for the enhancement of the statement. The result demonstrated that the existence of *LF*-containing plasmid (pVLFK, pVLFNp) and pVAL-1 was determined in each transconjugant (Figure 3.5). The human lactoferrin gene was detected in only strain pVLFK and pVLFNp by using PCR technique, with specific primers to amplify the 2.1 kb PCR product, identified as *hLF*. Moreover, sequencing data showed the correct subcloning site and promoter-like region of *hLF* in pVLFK, which assessed by GeneticMac software, and pVLFNp (Figure 3.2). The data analysis showed that the promoter like region of *hLF* gene in pVLFK was more than 50% similar to that of *E. coli*. The results approved that the *hLF* gene was stable and replicable in *B. uniformis* with erythromycin selection.

4.2 Human lactoferrin gene expression in *B. uniformis*

To determine the transcription of *hLF* in *B. unifomis*, the mRNA of *hLF* was detected by Northern blot analysis. Hybridization with an internal *hLF* probe showed that the mRNA of hLF was detected in *B. uniformis* strains BU1001 (pVLFK) and BU1001 (pVLFNp) (Figure 3.6), but not in *B. uniformis* strain BU1001 (pVAL-1). The results suggested that the *hLF* gene had successfully transcribed in *B. uniformis* using pVAL-1 as a expressing plasmid vector without any non specific hybridization. The transcriptional level of *hLF* was slightly lower in *B. uniformis* strain BU1001 (pVLFK) than in strain BU1001 (pVLFNp). This indicated that the higher transcriptinal level in strain BU1001 (pVLFNp) was caused from the function of the *nanH* promoter. The *nanH* promoter was successful in increasing the level of transcription in *B. uniformis*. Godoy *et al.*,(1993) indicated that the *nanH* promoter had been driven during the growth of *B. fragilis*. They suggested that neuraminidase activity might be required for the *B. fragilis* to grow to a maximal level in the tissue culture and rat pouch system by making other carbon sources available after glucose levels were reduced (Godoy *et al.*,1993). Fraser and Brown (1981) also reported that within the *B. fragilis* group of species, *B. fragilis, B. vulgatus, B. distasonis, B. ovatus, B. thetaiotaomicron* and *B. variablis* were neuraminidase positive, while *B. eggerthii, B. uniformis* and *B. splanchnicus* were negative. However, the *nanH* promoter had worked similarly in *B. uniformis*, when monitored by the increase of *hLF* mRNA. The mRNA was also detectable by using the C-terminal probe of *hLF* (Figure 3.6C). It indicated that the complete expression of *hLF* was processed. However the incomplete expression was also detectable by a smear tail from the LF mRNA bands. To avoid radioactive use, the nonradioactive DIG system was used. Digoxygenin (DIG) is a steroid hapten used to label DNA, RNA or oligonucleotide for hybridization and subsequent color or luminescence detection. This system, developed and marketed by Boehringer-Mannheim, typically employs hybridization probes labeled with digoxigenin, a plant alkaloid not normally present in clinical samples.The hybridized probes are immunodetected with anti-DIG-alkaline phosphatase. Because of the lower steps of the probe labeling procedure and remarkable sensitivity that could probe in a Southern blot of 1 μg DNA (Boehringer Mannheim Catalog: 1175 003), this method is suitable for labeling a probe for subsequent hybridization instead of radioactive labeling.

Although the transcriptional level of *hLF* mRNA was lower in *B. uniformis* strain BU1001 (pVLFK) than strain BU 1001 (pVLFNp), the specific gene product of the predicted size could be detected in the cell homogenate of only strain BU1001 (pVLFK), by Western blot analysis using rabbit anti-human lactoferrin antisera. To verify the presence of rhLF in the cell homogenate of *B. uniformis* (pVLFK), two detection systems of antibody-protein interaction were applied. The result of detection in those systems was similar. The result subsequently showed that rhLF was presented on the outer-membrane of *B. uniformis* strain BU1001 (pVLFK) as showes in Figuer 3.8. It revealed that the rhLF was located on the cell surface that might be the functional molecule for its biological activity. Unfortunately the *hLF* fused with *nanH* promoter (pVLFNp) could not produced rhLF in *B. uniformis* BU1001 detected by anti-hLF antibody. It might be that *hLF* gene in pVLFNp was not appropriate to translate for rhLF, such as the position of ribosome binding site. It was also possible that the recombinant protein which high production rate was aggregated to form inclusion body, which was not detectable by specific antibody. Therefore the detection system for rhLF in *B. uniformis* BU1001 (pVLFNp) should be improved. The low level of *hLF* gene expression might take advantage of the successful production of rhLF in *B. uniformis,* because lactoferrin had bactericidal activity, and the rate of its production must be minimized in prokaryotic statement systems to reduce toxicity to the host bacteria (Sitaram *et al.,*1998). Sritaram *et al.* (1998) reported the statement of bovine lactoferrin in *E. coli* by temperature control to avoid over-expression of the *bLF* gene. Majerle *et al.* (2000) produced recombinant isotopically enriched peptide and applied it to the production expressed human lactoferrin in *E. coli.* Peptides were produced in a high yield as fusion proteins with ketosteroid isomerase, which formed insoluble inclusion bodies. The authors suggested that the insoluble form allowed easy purification, stabilized the peptide against degradation and prevented bactericidal activity of the peptide. Wapp *et al.* (1992a) reported the production of recombinant lactoferrin in *Aspergillus oryzae* under the control of *A. oryzae* alpha-amylase promoter. Moreover, rhLF was also expressed in *A. nidulamts*, under the control of strong-ethanol inducible alcohol dehydrogenase promoter. The rhLF was produced at levels above 5-25 microgram/ml (Wapp *et al.,*1992b). Recombinant LF was secreted into the growth medium about 30%, while this study exhibited no secretion from cell to culture supernatant (Figure 3.7). The concentration of rhLF produced from *B. uniformis* in both cell free extract and culture supernatant failed to be

determined by ELISA with mouse anti-human lactoferrin monoclonal antibody. By this system, it was very difficult to detect rhLF in both fractions because various cellular proteins contained in the samples interfered with the binding of rhLF with a specific antibody. The detection of rhLF by ELISA methods, with a specific monoclonal antibody required some purification steps such as cation exchange chromatography (Yoshida *et al.*, 2000), affinity chromatography (Hutchens *et al*, 1989; Kawakami *et al.*,1987) or HPLC (Nagaoka and Maitani, 2000). Then pVLFK was completely analyzed for N-lobe human lactoferrin and deduced to amino acid sequence (Figure3.5). Sequence analysis monitored no mutation in the *LF* gene during replication and transcription in *B. uniformis*.

4.3 Bacteriostatic property of lactoferrin-producing *B. uniformis*

The biological activity of *B. uniformis* carrying the *hLF* gene was determined by the growth-inhibitory effect on *E. coli* strain HB101, *in vitro*, whereas the culture of *B. uniformis* BU1001 (pVLFK) showed a weak inhibitory effect on its growth *in vitro*, probably due to the presence of hLF (Table 2). Minimum inhibitory concentrations of bovine and human lactoferrin against *E. coli* were determined at 2 mg/ml and 3 mg/ml, respectively (Bellamy *et al.*,1992). The results also showed that human lactoferrin at a dose of 3 mg/ml in GAM broth did not exhibit the inhibitory effect on the growth of *E. coli* HB101. Experiments with human lactoferrin indicated that its membrane activity was related to an ability to directly interact with the membrane and protein that bind to the LPS molecule (Ellison *et al.*,1991). Then Yamauchi *et al.* (1993) found that the bovine protein shared these latter properties. The concentration of lactoferrin required to alter the outer membrane was high, indicating that these activities would not occur in all physiological environments. However, lactoferrin was found at the following levels in body fluids; 0.5 mg/ml in pooled pulmonary secretion, above 6.0 mg/ml in preterm colostrum and above 14 mg/ml in infected paratid fluid. Additionally, lactoferrin was released from PMNs in response to cytokine stimulation and gram-negative bacterial infection. Yamauchi and their colleagues also showed that bLF caused both the release of structural LPS molecules and an increased in the killing of bacteria by human lyzozyme (Yamauchi *et al.*, 1993). In this fashion, bLF appears to have an effect similar to that of human lactoferrin (Ellison *et al.*, 1991, 1988, 1990a, 1990b). These results suggested that the concentration of hLF in the culture of

B. uniformis BU1001 (pVLFK) might be too low to show sufficient bactericidal effect, or the hLF produced in the prokaryotic cells might lose some biological activity. One proposed mechanism on growth inhibition was the modification of culture medium by *B. uniformis* BU1001 (pVLFK). The culture of *B. uniformis* BU1001 (pVLFK) might change the composition to be inappropriate for the growth of *E. coli*. Because *E. coli* was selected as a β-glucuronidase-positive bacteria and associated with the high risk group for colorectal cancer (Swidsinski *et al.*, 1998), lactoferrin-producing *B. uniformis* could modulate the growth of *E. coli* in intestinal lumen. Then there is a possibility that the growth inhibition of *E. coli* might reduce the effect of this bacterium on the colon carcinogenesis.

4.4 Anti-carcinogenesis of lactoferrin-producing *B. uniformis*

It was proposed that azoxymethane-induced aberrant crypt focus (ACF) was a preneoplastic lesion for colorectal carcinogenesis (Bird, 1987). Quantification of the number and growth features of ACF was employed to study modulators of colon carcinogenesis (Bird, 1995). The total number of ACF and multiple ACF, with more than three crypts per focus, were significantly increased, about 45% in rats treated with the culture of *B. uniformis* BU1001 (pVAL-1). The result suggested that cellular components and/or metabolites of *B. uniformis* might enhance AOM-induced ACF formation in the rat colon. In this study, *Bacteroides spp.* was selected as a host to produce hLF because it has been predominant anaerobic microflora in the human colon. In the same manner, the results in Table 3.6 show that *B. uniformis* BU1001 also augmented the effect of AOM to induced the ACF formation in the rat colons. Additionally, preliminary data demonstrated that erythromycin contained in GAM broth (10 μg/ml) enhanced about 24% of the number of ACF in the AOM-treated rat colons, compared to that in rats fed with only water. This data also showed that rats fed GAM broth with AOM administration had a similar number of ACF to that in the water-treated group (Arimochi *et al.*, 1997). Erythromycin in a selective concentration might enhance the growth of some bacteria strains, which promote the growth of ACF, because the development of beneficial microflora strains was inhibited. Almost all *E. coli* species were resistant to a low concentration of erythromycin (10 μg/ml), which might cause an increase in the population of *E. coli*. This was found to associate with colorectal cancer in the high-risk group (Swidsinski *et al.*, 1998). Arimochi *et al.* (1999) constructed lycopene

producing *E. coli* and investigated the modulating effect on ACF formation in the rat colons. Their results showed that *E. coli* harboring pVAL-1 also increased the number of ACF in AOM treated rats of the control group (saline+AOM). They also suggested that other gene products expressed from pVAL-1 might affect colon carcinogenesis. Certain intestinal bacteria converted lipid to second messengers such as diacylglycerol (Morotomi *et al.*,1997) and long chain fatty acid (Morotomi *et al.*,1990). These messengers entered the colonic epithelium (Morotomi *et al.*,1991) and altered cell proliferation by activating protein kinase C (Carven *et al.*,1987). More recently, Onoue *et al.* reported that colonization of certain intestinal bacteria influenced the ACF profile in germ free rats treated with DMH (Onoue *et al.*,1997). Several strains of some *Bacteroides spp.* were reported to produce faecapentanes (Van Tassel *et al.*,1982). Zarkovic *et al.* indicated that faecapentanes promoted tumor and ACF development in rat colon carcinogenesis (Zarkovic *et al.*,1993). Despite an increase in the number of ACF by treatment with the culture of *B. uniformis*, a recombinant strain *B. uniformis* BU1001 (pVLFK) significantly decreased the number of total ACF when compared with that in the plasmid control group (Table 3.2). *B. uniformis* BU1001 (pVLFK) also slightly decreased the number of ACF of rats in the water group but any significance was not observed (Figure 3.11). In addition, the multiplicity of ACF was slightly decreased in the *B. uniformis* BU1001 (pVLFK)-treated group. These results indicated that *B. uniformis* produced human lactoferrin, which could inhibit the ACF formation in the rat colon, when synergistically induced by AOM, erythromycin and plasmid components. The number of aerobic, anaerobic and erythromycin-resistant anaerobic bacteria in the feces of *B. uniformis* BU1001 (pVLFK)-treated rats was slightly lower than that of water- and *B. uniformis* BU1001 (pVAL-1)-treated rats. This data indicated that the decrease of intestinal bacteria in the rat colon, through treatment with lactoferrin-producing *B. uniformis,* might relate to cell proliferation in the colonic mucosa. The profile of intestinal bacteria that affected on the ACF formation in AOM-treated rats, might be modified by the recombinant lactoferrin produced from *B. uniformis* BU1001 (pVLFK). Similar to human lactoferrin, bovine lactoferrin and lactoferricin, the pepsin-digested product showed inhibitory effects on the AOM-initiated colon tumor and ACF development (Tsuda *et al.*,1998). It was shown that the antitumoral activity of human lactoferrin might be explained by the modulation of the natural killer cell (NK) and lymphokine-activated killer cell cytotoxicity (Shau *et al.*,1992). The anti-neoplastic activity of lactoferrin also

seems to be due to the direct action on the target cell (Damiens *et al.*,1998). Damiens *et al.*,(1999) additionally demonstrated that lactoferrin inhibited the proliferation of the transformed and tumor epithelial cell lines by a block in the cell cycle progression at the G1 to S transition . The extension and modification of the cell cycle could explain the increase in sensitivity to the lysis of transformed and tumor epithelial cells (Damiens *et al.*,1998). Because of the multifunction of lactoferrin, the bacteria-produced lactoferrin could be used as a functional bacteria for a colon cancer prevention strategy, although *B. uniformis* BU1001 harboring pVAL-1 could enhance the number of ACF in AOM treated rats.

Bifidobacteria spp. are frequently reported as one of the predominant organisms found in the feces of breast fed infants. More importantly, *Bifidobacteria spp.* are believed to be a beneficial component of the indigenous flora in the infant because of their ability to produce acetic and lactic acid which may discourage pathogenic bacteria (Bullen and Tearle, 1976, Modler *et al.*, 1990). Human and bovine lactoferrin promote the growth of intestinal bacteria of the genus Bifidobacterium *in vitro* by a mechanism that apparently does not require binding of the LF molecule to the bacterial surface. The growth promotion activity of LF for *Bifidobacteria spp.* does not appear to be related to the acquisition. Utilization of iron and LF from human milk has shown a greater promotion of activity for *B. bifidum*, while bLF has been more active for *B. infantis* and *B. brave* (Petschow *et al.*, 1999).

The deconjugation reaction might drive from different enzymes produced from bacteria in other erythromycin-resistant strains. *Bacteroides spp.* was reported as the intestinal bacteria produced β-glucuronidase. Morotomi *et al.* (1985) reported that the cell free extract of some strains of intestinal bacteria (including *B. fragilis, B. vulgatus, B. thetaiotaomicron, Eubacterium eligens*, *Peptostreptococcus* and *E. coli*) hydrolyzed the synthetic β-glucuronide of phenolphthalein and/or p-nitrophenol. In this study, *B. uniformis* in both strains BU1001 (pVAL-1) and BU1001 (pVLFK) did not exhibit β-glucuronidase activity in either cell free extracts or culture supernatant (Table 3.4). Another possibility concerning the inhibition of carcinogenesis might be through its action on bacteria and related enzymes. Reduction of β -glucuronidase was observed after milk and *L. acidophilus* feeding (Goldin and Gorbach, 1984). β-glucuronidase has been an enzyme responsible for releasing methylazoxymethanol from the glucuronide conjugated form. This would be expected to reduce the initiating potential of administered carcinogens and

also the generation of DNA damage in tumors, which would inhibit their progression. The results from this study showed that the activity of β-glucuronidase was not related to ACF formation in rats treated with AOM. Rats fed with the culture of bacteria containing erythromycin showed a significantly lower activity of β-glucuronidase (Table 3.5). The effect of antibiotic might kill some population of β-glucuronidase-producing strains and increase other populations. The number of aerobic bacteria was similar in each group, while anaerobic bacteria in the feces of rats fed with bacterium culture were higher than that of the water group (Table 3.3). The results monitored that β-glucuronidase did not play a main role in the AOM-induced colon carcinogenesis.

The human colon contains a complex bacterial population, of which over 99% are obligate anaerobes. One numerical colonic anaerobe is Bacteroides, which is also an opportunistic pathogens that can cause serious infections in humans if it escapes from the colon, due to abdominal surgery or other trauma. Moreover, previous data showed that *B. uniformis* containing pVAL-1 increased the number of ACF in AOM-treated rats (Table 3.2). Then the effect of *Bacteroides spp.* on the formation of ACF in the rat colon was investigated. The strains used in this study were made available in the laboratory and included in almost all strains of *Bacteroides spp.* identified in 1994 (Paster *et al.*, 1994). The results showed that the *Bacteroides spp.* consisted of both an increased and decreased number of ACF and they also had no effect on the ACF formation. This is the first report that showed the effect of an individual strain of *Bacteroides spp.* on AOM-induced ACF formation. Moore and Moore (1995) expected that the *Bacteroides spp.* were associated with the high risk of colon cancer because the consumption of red meat (Moore and Moore, 1995) and a high fat-diet was reported to be associated with this disease and fat stimulates flow, which in turn specifically stimulates *Bacteroides spp.* (Draser and Hill, 1974, Finegold, 1977). Only two *Bacteroides spp.*, *B. vulgatus* and *B. stercoris,* showed a significant association to a high risk, as opposed to low risk (Moore and Moore, 1995). However *Bacteroides spp.* showed high percentage in the high-risk group. The result in this study showed that *B. distasonis*, *B. ovatus* and *B. uniformis* BU1001 were species that enhanced the number of ACF formation. This might be different from isolated sources. The result suggested that within the ACF decreasing group, unlike *B. uniformis* BU1001, *B. uniformis* KYU 2 showed some inhibitory effects on the formation of ACF. Moreover, this strain is in the same species of

B. uniformis BU1001. Therefore, *B. uniformis* KYU 2 was selected to produce lactoferrin and investigate the modulating effect on AOM-induced ACF formation in the rat colon.

Two independent experiments using *B. uniformis* KYU 2 were performed to investigate the modulating effect on AOM-induced ACF in the rat colon. No any significant difference within the AOM-treated group was observed. It might be interpreted that the *hLF* gene could not be expressed in the *B. uniformis* strain KYU 2. The expression level of transcription and translation needs further investigation. After this experiment, *B. uniformis* KYU 2 was identified as a *B. fragilis* strain by genetic analysis (Kuwahara *et al.*,unpublished data, which suggested that it was resistant to erythromycin. The stability of pVAL-1 was lower in *Bacteroides spp.* than *E. coli* (Valentine *et al.*,1989), without the selection of erythromycin. Therefore, the *B. uniformis* KYU-2 harbored pVLFK might be decreased when the serial inoculation for bacterial culture is operated. Valentine *et al.* also approved that after 20 generations of *Bacteroides spp.* transconjugants, only 2-4% of bacterial colonies were Em-resistant. The non-transconjugant also grew instead of pVAL-1-harboring *B. uniformis*. The results of this study suggested that *B. uniformis* KYU-2 (*B. fragilis*) could not be applied as a host for the expression of the recombinant protein by using erythromycin as a selection marker. The new suitable strain of Bacteroides will be investigated further for the expression of rhLF.

In conclusion, the *hLF* was successfully expressed in other prokaryotic systems over *E. coli* or *Aspergillus* by using *B. uniformis* as a host for expression. The *hLF* mRNA could be hybridized by *hLF* cDNA probe (transcription) and the rhLF could be recognized by anti-hLF antibody (translation). The lactoferrin-producing *B. uniformis* could retard the growth of *E. coli* HB101 *in vitro*. It might alter the growth of intestinal bacteria, which played a role in AOM-induced colon carcinogenesis. Lactoferrin-producing *B. uniformis* modulated the AOM induced ACF formation in the rat colon, although *B. uniformis* strain BU1001 or KYU-2 increased the number of ACF in it. This effect did not throughout the bacterial β-glucuronidase activity. The proposed mechanisms of lactoferrin-producing *B. uniformis* are to control the growth of pathogenic bacteria such as *E. coli* and/or modified the environment of the large bowel. The trace amount (undetectable) of rhLF or digested LF produced from *B. uniformis* BU1001 (pVLFK) might modulate continuously in the immunity of the colon. These results supported the hypothesis that to reduce harmful bacteria is one strategy to prevent colon carcinogenesis. These

results lead to an additional construction of lactoferrin-producing intestinal bacteria by using the strains of Lactobacillus, Bifidobacterium and Lactococcus as a host for statement. These are known to reduce carcinogen-induced ACF formation in the rat colon and promote host health (Gallaher *et al.,* 1996; Singh *et al.,*1997; Holzapfel *et al.,*1998).

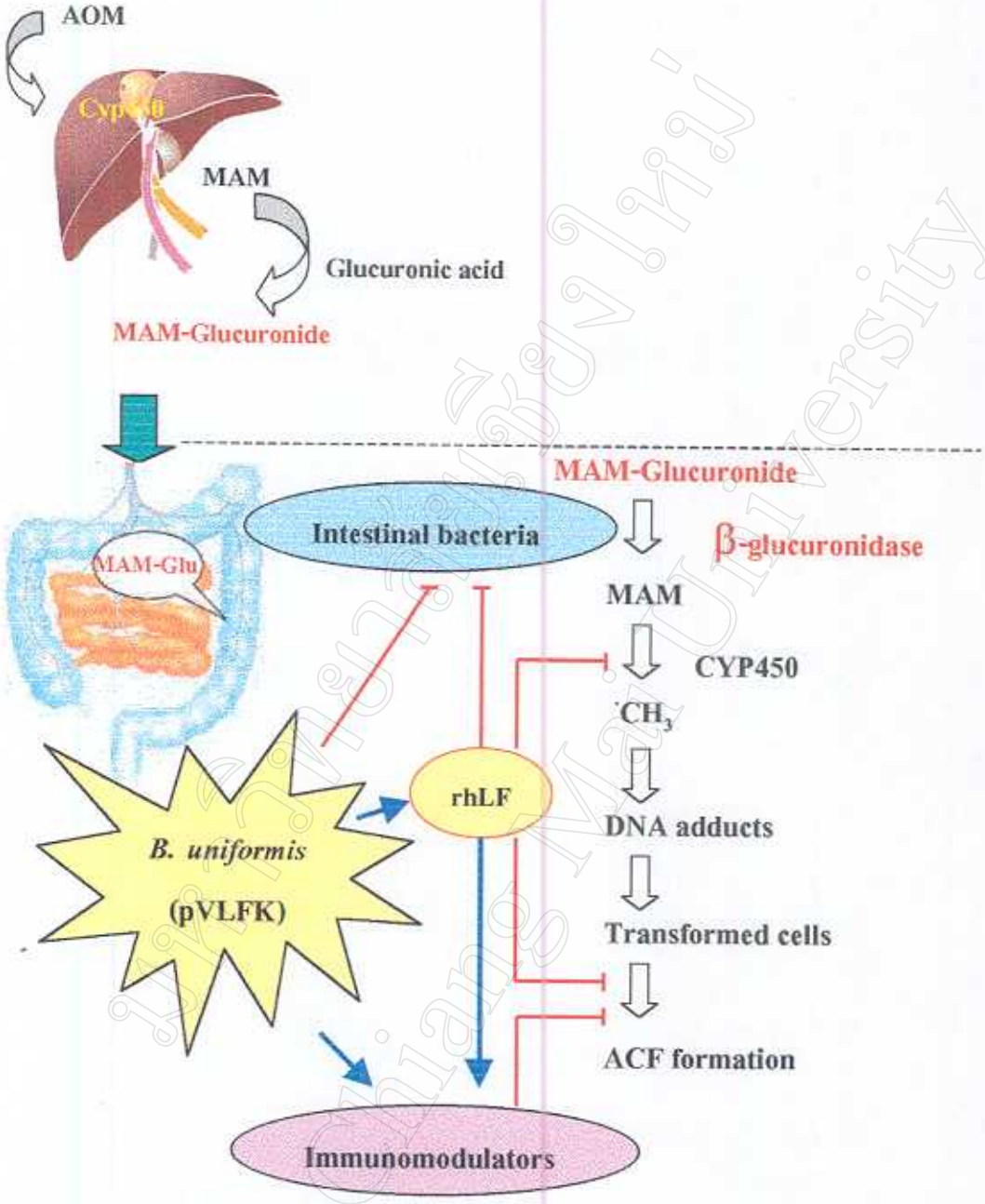

Figure 4.1 Proposed mechanism of lactoferrin producing *B. uniformis* on AOM-induced colon carcinogenesis.