โครงการ
การสร้างเชื้อ recombinant *Bifidobacterium breve* ที่มีการแสดงออกของโปรตีน viral capsid protein1 (VP1)

โดย

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1. บทคัดย่อ

รหัสโครงการ : MRG5680081

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เอนเทอโรไวรัส 71 (enterovirus 71, EV71) เป็นสาเหตุของโรคมือเท้าปากในเด็ก การติดเชื้อ EV71 มักจะเกี่ยวข้องกับภาวะแทรกซ้อนทางระบบประสาทอย่างรุนแรงและส่งผลให้มีอัตราการเสียชีวิตสูง ปัจจุบันยังไม่มีวัคซีนที่มีประสิทธิภาพเพื่อใช้ป้องกันการติดเชื้อ EV71 ดังนั้นจึงจำเป็นต้องมีการพัฒนาวัคซีนสำหรับป้องกันการติดเชื้อไวรัสชนิดนี้ การศึกษาการตอบสนองระบบภูมิคุ้มกันต่อ EV71 พบว่าองค์ประกอบของไวรัสคือ viral capsid protein 1 (VP1) มีคุณสมบัติเป็นแอนติเจนที่ดีสำหรับกระตุ้นการสร้างแอนติบอดีชนิดลบล้างฤทธิ์ได้ แบคทีเรียกลุ่ม *Bifidobacterium* spp. มีคุณสมบัติเป็นโปรไบโอติกส์ที่ดีและสามารถนำมาดัดแปลงเป็นตัวนำส่งแอนติเจนในการพัฒนาเป็นวัคซีนชนิดกิน งานวิจัยนี้มีวัตถุประสงค์เพื่อสร้างเชื้อลูกผสม *Bifidobacterium breve* UCC2003 ที่มีการแสดงออกของ VP1 แต่การศึกษาช่วงเริ่มต้นพบว่าไม่สามารถนำพลาสมิดลูกผสมเข้าสู่เชื้อนี้ได้ จึงทำการเปลี่ยนเซลล์แบคทีเรียเจ้าบ้านเป็นเชื้อโปรไบโอติก *Bifidobacterium pseudocatenulatum* M115 ผลการศึกษาพบว่าสามารถสร้าง recombinant expression plasmid ที่ควบคุมการแสดงออกของ VP1 ภายในเซลล์ของเชื้อ *B. pseudocatenulatum* M115 ได้ โดยการแสดงออกอยู่ภายใต้การควบคุมของ P919 constitutive promoter และ transcriptional terminator การทดสอบการแสดงออกของ VP1 โดยวิธี western blot analysis พบว่า recombinant expression plasmid สามารถควบคุมการสร้างโปรตีน VP1 ภายในเชื้อ *B. pseudocatenulatum* M115 ได้ อย่างไรก็ตามงานวิจัยครั้งนี้ยังไม่ประสบความสำเร็จในการสร้างเชื้อ *B. pseudocatenulatum* M115 ที่มีการแสดงออกของโปรตีน VP1 ในส่วนภายนอกเซลล์และฝังตัวที่ผนังเซลล์แบคทีเรีย ซึ่งผู้วิจัยยังคงได้ดำเนินงานส่วนนี้ต่อไป

คำสำคัญ : Enterovirus71 viral capsid protein 1 (VP1), Oral live vaccine, Lactic acid bacteria, *Bifidobacterium pseudocatenulatum*

1. Abstract

Project Code : MRG5680081

Project Title : Construction of recombinant *Bifidobacterium breve* expressing enterovirus71 viral capsid protein 1 (VP1)

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Enterovirus 71 (EV71) is causative agent of hand, foot, and mouth disease in young children. Infection with EV71 has been often associated with severe neurological complication resulting in high mortalities rate. Nowadays, no effective vaccines against EV71 infection have been generated. Therefore, further vaccine development against EV71 infection is needed. The previous study of immune response to EV71 found that the viral capsid protein 1 (VP1) of EV71 has been shown to induce the protective neutralizing antibodies and thus it can be used in vaccine formulation. The bacterial *Bifidobacterium* spp. have been considered as probiotics and some strains of these bacteria have been used as antigen delivery vaccine for oral vaccination to elicit mucosal immune system against EV71 infection. This study aim to construct the recombinant *Bifidobacterium breve* expressing EV71-VP1. However, attempts to introduce the recombinant plasmid into *B. breve* UCC2003 were unsuccessful. For this reason, the expression host was changed from *B. breve* UCC2003 to the probiotic *B. pseudocatenulatum* M115. The result found that the new recombinant expression plasmid containing VP1 gene under the control of P919 promoter and transcriptional terminator was successfully constructed. Based on western blot analysis revealed that the recombinant expression plasmid could control the expression of EV71-VP1 in intracellular location of recombinant *B. pseudocatenulatum* M115.

Keywords : Enterovirus71 viral capsid protein 1 (VP1), Oral live vaccine, Lactic acid bacteria, *Bifidobacterium pseudocatenulatum*

2. Objectives

2.1 To select the promoter for the expression of VP1 in *B. breve* UCC2003.

2.2 To develop secretion vector for the expression of VP1 in extracellular location of *B. breve* UCC2003.

2.3 To characterize of anchoring sequences from bifidobacteria, to attach the VP1 into cell wall of *B. breve* UCC2003.

3. Background and rationale of the study

Enterovirus 71 (EV71) is the virulent viral type of hand, foot, and mouth disease (HFMD) in infant and young children. EV71 is a member of the *Picornaviridae* family [1]. EV71 infection is often associated with neurological complication, and resulting in high morbidities and mortalities. Various types of vaccine against EV71 are being investigated including inactivated whole viruses [2], live attenuated virus [3], viral-like particle [4], subviral particle, and DNA vaccine [5]. These vaccines showed a high efficacy in the induction of a specific immune response, especially serum neutralizing antibodies against EV71. However, the efficacies of these reported vaccines against EV71 infection in animal and human have not been described. Thus, the further alternative vaccine development against EV71 infection is needed. Four viral capsid proteins (VP), i.e. VP1, VP2, VP3, and VP4 of EV71 have been shown to be good immunogens. Among all these, the VP1 is the most potent immunogen, as it has been demonstrated to induce neutralizing antibodies to prevent infection [6]. Therefore, VP1 can be used as candidate antigen for incorporating in vaccine formulation. Comparing with other routes of vaccination, the parenteral route is the effective route to elicit protective systemic immunity, but it often lacks of localized immunity, where most pathogens, including Enterovirus71, enter the body. Mucosal vaccination offers several advantages, including needle-free injection, preventing the initial infection at the site of entry, stimulating both local and systemic immune responses, ease of administration and low delivery costs [7].

Many strains of bacteria in the genus *Bifidobacterium*, a member of lactic acid bacteria, have been considered as probiotic. They are able to survive and colonize in the gastrointestinal tract of human [8], to modulate the immune response, to inhibit infection by certain pathogens [9], and to enhance anticarcinogenic activity [10]. They are one of several attractive candidates used for developing oral vaccines to elicit mucosal immunity against EV71 infection. Recombinant bifidobacteria expressing heterologous reactive antigens have been constructed and used as prophylactic and therapeutic vaccines [11, 12]. Some gene cloning and expression systems have been already developed in bifidobacteria. Therefore, using bifidobacteria as antigen delivery vehicle for oral vaccination is a challenging approach in modern vaccinology.

In this study, we aim to construct the recombinant plasmid containing VP1-encoding gene for three cellular locations, e.g. intracellular, extracellular and cell-wall anchored" of *Bifidobacterium* sp.. Our results revealed that only intracellular expression of VP1 in *Bifidobacterium pseudocatenulatum* M115 was obtained. For extracellular and cell wall expression, some plasmid vectors, e.g. secretion-expression vector has been already constructed, but the protein verification has not been successfully finished in this project. To finish this aim, further verification of VP1 is required. Finally, The recombinant *B. pseudocatenulatum* M115 expressing VP1 protein in intracellular might be valuable tools for the further evaluation of their immunogenicity in eliciting the immune response in animal model.

4. Literature review

4.1 Enterovirus 71

4.1.1 Virology

Enterovirus 71 (EV71), a small, non-enveloped virus, belongs to genus *Enterovirus,* family *Picornaviridae* [1]. Its genome is positive single-stranded RNA (+ssRNA) with the length of approximately 7.5 kilobases and consists of a single open reading frame (ORF) that encodes a polyprotein consisting of P1, P2 and P3 regions. P2 and P3 regions encode seven non-structural proteins while P1 region encodes four viral structural proteins VP1, VP2, VP3 and VP4 [13]**.** VP1, VP2, and VP3 are exposed outside of the viral capsid, whereas VP4 is located inside the virus capsid (Fig.1). Among viral capsid proteins, the VP1 of EV71 contains neutralization epitopes which are interested for use and developing as subunit vaccines against EV71 [6].

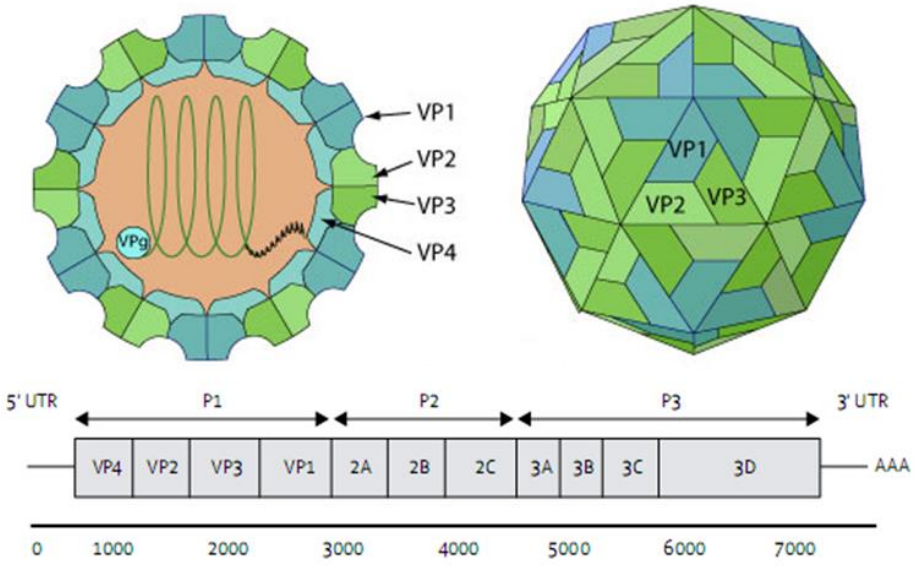


Figure 1 Schematic representation of EV 71. The viral capsid consists of four structural proteins, i.e., VP1, VP2, VP3 and VP4. The VP1-VP3 proteins are exposed on the virion surface, while VP4 protein is located on the internal side of the capsid. Viral genomic RNA has a viral protein (VPg) at its 5' end instead of a methylated nucleotide cap structure. The long UTR at the 5' end contains a type I internal ribosome entry site. The polyprotein is initially processed by the viral proteases into three regions, P1, P2, and P3. The P1 region encodes the structural polypeptides, VP1-VP4 as described above. The P2 and P3 regions encode seven non structural proteins associated with replication.

4.1.2 Pathogenesis and epidemiology

EV71 is the causative agent of hand, foot and mouth disease (HFMD) in infant and young children younger than 5 years old. The disease is characterized by vesicular lesions on the palms, soles and oral mucosa. The disease usually shows a mild symptoms and recovers without medical treatment within 7 to 10 days [5]. Some

young children developed severe neurological syndromes, such as polio-like paralysis, fatal encephalitis and even death. The disease can be transmitted by contact with fecal contaminated materials (fecal-oral route) or by stool touching, respiratory droplets or respiratory secretions and herpes solution [14]. EV71 was firstly isolated in 1969 in California. The outbreaks of EV71 with significant morbidities and mortalities have been reported worldwide, especially in Asia-Pacific region [15, 16]. Based on VP1 sequence, EV71 can be divided into genotypes A, B and C. Genotypes B and C are separated into subgenotypes B1-B5 and C1- C5, respectively. Since 1997, EV71 outbreaks occurred in Sarawak, Malaysia [17, 18], Taiwan [19], Singapore [20] and Vietnam [21]. In 2008, there are more than 6000 HFMD cases and 22 deaths in China. The last outbreak of EV71 has been reported in Cambodia with 52 out of 59 confirmed cases have died. At present, the means of primary prevention and control of EV71 spreading is by public health surveillance and quarantine. In the near future, it has been predicted that EV 71 will become a new emerging neurotropic enterovirus and a threat to global public health and the EV71 infection would increase in many countries, including Thailand. In order to prevent the infection, the most effective strategy is vaccination. However, up to date there is no effective vaccine and antiviral treatment for EV71 infections.

4.1.3 Viral antigen involving in immunity

After infection, the primary immune response against an infection is CD4+ T cell-dependent and the induction of a cytotoxic cellular response including the efficient antibody response.

Three amino acid regions presented on VP1 protein, including region 66–77, 145–159, and 247–261, were identified as the epitopes for the induction of CD4+ T cells proliferation and IL-2 and IFN-γ production. Among these three regions, amino acids 145–159 induced the strongest T cell proliferative response and highest cytokine production [22]. A recombinant VP1 protein with complete Freund's adjuvant

can elicit a neutralizing antibody response, enhance T helper cell proliferation, induce high levels of interleukin (IL)-10 and interferon (IFN-γ) in mice [6]. EV71 VP1 protein can be expressed in *B. longum* to generate mucosal and systemic immune responses in mice [23]. In addition, the VP1-specific fecal IgA and serum IgG were observed in mice, and both humoral and cellular immunity against EV71 were established [24]. The VP1 sequences are highly conserved within the different sub-genotypes and the identification of more T-cell and B-cell epitopes of the VP1 protein have been observed.

4.2 Vaccine

4.2.1 Vaccine development against EV71

As mention above, there is no effective vaccine and antiviral treatment against EV71 infection. Recently, several EV71 vaccine candidates are being developed including inactivated whole virus [2], live-attenuated virus [25, 26], virus-like particle, recombinant VP1 protein [27, 28] and DNA vaccines. The efficiency of these vaccines has been assessed in animals, but no clinical trial has yet been conducted. For the live-attenuated virus, the success of live-attenuated vaccine in preventing poliomyelitis and eradicating the polioviruses indicates the potential for preventing EV71 by vaccination [25] and the cDNA-derived virulent and attenuated strains of EV71 should be an important tool for the elucidation of EV71-induced severe neurological disorders and development of a vaccine strain [26]. The virus-like particle (VLP) vaccine can preserved the conformational epitopes, has no risk of virulent revertants, and is one promising vaccine candidate in preventing EV71 infection [29]. The genetic immunization with DNA vaccine encoding VP1 gene of EV71 found that the VP1 DNA vaccine candidate induce immune response of serum neutralizing antibody in mice. In further study and improvement, use of the DNA vaccine might be a potential vaccine strategy against EV71 [5]. In addition, the monoclonal antibody generated by immunizing mice with amino acids 208–222 of VP1 showed strong neutralizing activity against EV71 in an *in vitro* neutralization assay. Therefore, the peptide vaccine

represents a promising candidate for EV71 [30]. The neutralizing antibodies against EV71 is the most important strategy in limiting the severity of infection [31]. Belong to the viral capsid protein VP1 to VP4, VP1 protein represents the major target for vaccine development because it contains a number of important neutralizing epitopes [32]. Thus, the VP1 seems to be the most potential antigen for EV71 vaccine development.

4.2.2 Lactic acid bacteria as live bacterial vaccine

Vaccination represents the most effective tool to prevent an infection [7]. Compare with the parenteral route of vaccination, the mucosal route is an approach to achieve full prevention by administration of vaccine, e.g., prevention of the initial infection and replication of the pathogens at the site of entry, stimulation both mucosal and systemic immune responses, easy to administration and low delivery costs [7]. Live bacterial vaccine is an attractive vaccine strategy use to induce an immune response to the bacteria itself or to carry vaccine component. The advantages of live vaccine include mimic route of entry, stimulation of the mucosal immune response, having intrinsic adjuvant properties and administration through oral or nasal route. Attenuated bacteria and food-related lactic acid bacteria were developed as live vaccine. Three live bacterial vaccines based on attenuated pathogens, i.e. *Mycobacterium bovis* BCG, *Salmonella* Typhi and *Vibrio cholera* have been used as vectors to express heterologous antigens [33]. However, use of attenuated pathogens as live vaccines has safety concerns involving risk of reversion to the virulent organisms or the possibility of causing disease in immunocompromised individuals [34]. To overcome these problems, non-pathogenic bacteria especially lactic acid bacteria (LAB) offer an alternative as live vaccine, as they are generally recognized as safe [35] by being consumption for a long time in a large amounts without causing any known health problem.

4.3 Lactic acid bacteria

LAB are a group of gram-positive bacteria, non spore forming, acid tolerance, ferment carbohydrate and produce lactic acid as the major end product [36]. In addition, some of them can produce acetic acid, bacteriocins and ethanol [37]. The members of LAB consist of a wide range of bacterial genera including *Pediococcus, Enterococcus, Lactococcus, Lactosphaera, Leuconostoc, Lactobacillus, Streptococcus, Tetragenococcus, Carnobacterium, Melissococcus, Oenococcus, Vagococcus, Weissella, Microbacterium, Aerococcus* and *Bifidobacterium* [38-40]. LAB have a generally recognized as safe (GRAS) status and widely play an important role in the food fermentation and preservation of fermented products. LAB has also shown other benefits such as modulating of the immune system, reducing symptoms of lactose intolerance and reducing risk of cancers [41]. Recently, the use of LAB for production of various heterologous proteins for biotechnology, medical and veterinary applications is under active study [42].

4.4 *Bifidobacterium* species

4.4.1 Bacteriology of *Bifidobacterium* species

Bifidobacterium are the group of LAB. Most of them are anaerobic though some species can tolerate oxygen [43]. They produce mainly lactic and acetic acid as metabolic end-products. *Bifidobacterium* are high guanine (G) and cytosine (C) DNA content bacteria. Members of the genus *Bifidobacterium* belonged to the phylum *Actinobacteria* and order *Bifidobacteriales. Bifidobacterium* strains commonly present in human and animal intestines and have been recognized as probiotic because of their beneficial effects in human, such as maintenance of the balance of human normal intestine flora [8], modulating the immune response [44], inhibiting pathogen infection by competing the colonization at the mucosal surface, inhibitory effect on colon cancer e.g. by modulation of colonic cell proliferation in rats [45], and protection against virus infection [46]. Currently, more than 40 species have been recognized as members of

Bifidobacterium, such as *B. pseudocatenulatum,* the dominant bifidobacteria strain in human gastrointestinal tract, *B. adolescentis*, *B. angulatum*, *B. breve*, *B. bifidum*, *B. catenulatum*, *B. dentium*, *B. infantis*, and *B.longum* [47]. Recently, the heterologous gene expression has been developed in bifidobacteria. It has been shown that the vector contained a heterologous genes could be control the expression without structural instability in *Bifidobacterium* strains [48]. However, to obtain the maximum production, bifidobacteria strains need to be genetically modified to improve native properties, introduce desirable characteristics and novel phenotypes, or remove undesirable traits.

4.4.2 Genetic of *Bifidobacterium* species and its applications

The genetic technologies of bifidobacteria remains poorly understood when comparing with other LAB [49]. However, due to the increasing number of available bifidobacteria genome sequence, the understanding of its genetic, physiology and evolution are being improved. Eleven bifidobacteria complete genomes have been sequenced and reported. Analysis of bifidobacteria genome have indicated that they contain a number of plasmids. Currently, 27 sequenced bifidobacteria plasmids have been reported, sixteen plasmids have been characterized from strains of *B. longum*, three from strains of *B. breve,* two from *B. bifidum* and *B. asteroids,* and one from *B. indicum* [50], *B. globosum*, *B. catenulatum* and *B. pseudocatenulatum*. The plasmids of bifidobacteria have the size ranging from 1.8 kb to 10.2 kb [51]. To develop the cloning vector for modification of bifidobacteria, understanding of the replication mechanism, genomic data of bifidobacteria and the efficient genetic tools are necessary [52]. As the gene manipulation and vector amplification in *Escherichia coli* is much easier than in bifidobacteria, thus shuttle vector for bifidobacteria and *E. coli* are often required. *Bifidobacterium* have provided the basis for the construction of *E. coli-Bifidobacterium* shuttle vector, i.e. pBC1 from *Bifidobacterium catenulatum* L48 [53]. The pBC1-based cloning vectors provide versatile genetic tool and adding new possibilities for cloning

and expression in strains of dominant bifidobacteria species in the human gastrointestinal tract (GIT) because it has high stability of both segregation and structure stability. Antibiotic resistance genes are the common selective markers use to construct a cloning vector in *Bifidobacterium* such as the ribosome-protection tetracycline resistance determinant gene *tet*(W) and the chloramphenicol-acetyltransferase gene (*cat*) [54-56]. In addition, other genetic tools including transformation system (electrotransformation and conjugation) [51], constitutive and inducible expression system [57, 58], targeting-expression system (intracellular, extracellular and cell wall anchored location) have been developed [59]. Construction of vector based on bifidobacterium replicon has been reported. The pAM1 vector, the pBC1 replicon was cloned into pUC19E at *Bam*HI site, has a wide host range as it can replicate in many bifidobacteria species such as *Bifidobacterium breve* UCC2003 and *B. pseudocatenulatum* M115 [53]. The copy number for pAM1 was 30 copies in *B. pseudocatenulatum* M115. Therefore, pAM1 is one example of the vector able for cloning and expression in bifidobacteria.

4.4.3 *Bifidobacterium* constitutive promoter

The process of gene expression needs the strong promoters. Lactic acid bacteria promoters, one report has shown that the strengths of putative promoter derive from the chromosomal DNA of *L. lactis* LM0230 were successfully determined the luciferase gene in *E. coli* and *L. lactis* and these promoter can be used for the construction of food-grade expression vector to produce heterologous protein in *L. lactis* [60]. However, no such report for *Bifidobacterium*. Many studies have shown that the -10 and -35 region sequences affect promoter strength [61]. Some research showed strongly conserved TG motif preceding the 10 element were observed in *Bacillus* [61, 62]. The motif is weakly conserved in *E. coli* [63] but more strongly conserved in many Gram-positive bacteria [64, 65]. In additional, previous studies have indicated that the total RNA from *B. bifidum* BGN4 were selected through Microarray analysis revealed 7

genes showing relatively high-level expression among 1,000 genes. The -16 region TG motif of the 7 promoters of *B. bifidum* BGN4 was found -6 bp upstream of the -10 sequence. Seven putative promoters from the *B. bifidum* BGN4 were then cloned and were analyzed by the measurement of β-glucosidase activity in both *B. bifidum* BGN4 and *E. coli* DH5α. The enzyme activities were showed the strongest in P919 and P895, while P644 and P1527 microarray showed stronger than P919 and P895 and the expression levels at both the translational and the transcriptional stages were considered to screen strong promoters. The analysis growth rate of *B. bifidum* BGN4 associated promoter activity which the strong promoters did not inhibit the growth of *B. bifidum* BGN4. The analyzed of all putative promoter sequences that showed homology with P919 were observed in *Bifidobacterium* species [58].

4.4.4 Expression of heterologous protein in *Bifidobacterium*

Several heterologous genes have been successfully cloned and expressed in bifidobacteria. *Bifidobacterium longum* biovar *longum* with a vector pGBL8b containing the insect luciferase gene (*lucGR*) from a *Pyrophorus plagiophthalamus,* which insect luciferase is widely used as a reporter gene including genes from gram-negative , gram-positive bacteria [66] and several human genes [67]. Heterologous expression of cholesterol oxidase from *Streptomyces coelicola* in *Bifidobacterium longum* MG1 based on the 16S rRNA gene promoter of genus *Bifidobacterium* has been reported [68]. Also, three heterologous genes, *Bacillus licheniformis* α-amylase, *Pseudomonas florescens* lipase and *Streptomyces* sp. cholesterol oxidase genes have been cloned into pDG7 based on the pMB1 replicon of *Bifidobacterium longum* [53]. Analysis of protein expression and secretion in bifidobacterium is an essential tool for heterologous gene expression. A study on protein secretion, seven signal peptides are heterologous for protein export in *B. breve* UCC2003 [59]. The *B. longum* XynF signal peptide fused with human OXM sequence was successfully used to express and export recombinant human oxyntomodulin and interleukins 10 and 12. Construction of binary expression vector (pLR) containing hup promoter and *Bifidobacterium* ß-galactosidase signal peptide was used for production of the human interleukin-10 (hIL-10) in both *Escherichia coli* and *Bifidobacterium longum* [69]. Furthermore, a secretion vector pBESAF2 containing the α-amylase gene amyB secretion signals from *B. adolescentis* INT57 can be expressed in *B. logum* MG1 [68, 70]. This plasmid system was used for the expression and secretion of pediocin PA-1 from *B. longum* MG1 by using α-amylase from bifidobacterium [71]. This approach may be able to improve the way for heterologous expression of extracellular proteins and development of expression and secretion systems for genus *Bifidobacterium*.

4.5 Protein targeting system

The final location of expressed recombinant protein is an important to the type of immune response and the stability of the antigen expressed. The antigen expressed by genetically modified lactic acid bacteria (GM-LAB) could be localized as intracellular, extracellular or cell-wall anchorage form [35]. The protein expression in cytoplasm requires the expression vector containing ribosome binding site, the promoter and the start of open reading frame for protein synthesis while the expression of either the secreted or anchored protein required the signal peptide (SP) for its translocation across the plasma membrane. Several SP have been used for protein expression in LAB such as the SP of the fibrillar surface protein M6 of *Streptococcus pyogenes* [72, 73], the SP of an α-amylase reporter under the control of secretion signals from *L. casei* [74] and the usp45 from the main secreted protein in *L. Lactis*. All of these SP were based on Sec-dependent machinery for protein translocation across the membrane and Sortase-dependent machinery for protein anchorage to the cell wall [75].

4.6 Development of *Bifidobacterium* species as live oral vaccine

Non-pathogenic lactic acid bacteria that supports their safety and the ability to colonized region of the human gastrointestinal tract, especially bifidobacteria is the potential bacteria for use as an antigen expression vector for presenting the antigen to the intestinal mucosa [76]. *Bifidobacterium* are a promising delivery system into the human intestinal tract for other useful gene products, such as antigens, in live vaccine development [42]. The development of heterologous gene expression system in bifidobacteria has been reported [67].

In vitro production of antigens localizing in cytoplasm, on cell wall and extracellular location have been studied in LAB [75]. Several studies have demonstrated that secreted antigens can induce protective immunity that is uptake by lymphocytes [77-80]. For anchorage antigens may be stimulate directly with immune cell by antigen-presenting cells [81, 82]. However, the only expression system for the

intracellular and extracellular location of heterologous protein have been constructed in bifidobacteria [52]. Recently, SP of alpha-amylase from *Bifidobacterium adolescentis* INT57 has been successfully used to construct the secretion vector for the secretion of *E. coli* phytase *into* culture medium. Seven SPs have been identified from the genome of *Bifidobacterium breve* UCC2003 and utilized for the construction of secretion vector. Thus, it is interested to use these SPs for exporting other proteins such as viral protein and tumor suppressor protein.

5. Materials and methods

5.1 Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1.

Bifidobacterium sp. was cultured under anaerobic conditions at 37°C in MRS broth supplemented with 0.25% cystein (MRSC). *Escherichia coli* strain was grown at 37°C in Luria-Bertani (LB) broth with vigorous shaking. Antibiotics were added to the appropriate media 100 μg/ml ampicillin for *E. coli* and 2.5 μg/ml erythromycin for bifidobacteria.

Table 1 Bacterial strains utilized in this study

Strains	Relevant properties	Source or reference
Bifidobacterium breve UCC2003	Transformation host	Douwe Van Sinderen[a]
Bifidobacterium pseudocatenulatum M115	Transformation host	IPLA
Bifidobacterium longum C35	Source of cell wall anchorage sequence	IPLA
Escherichia coli XL1-blue	Transformation host	RBC Bioscience

[a]Assoc. Prof. Douwe Van Sinderen, Department of Microbiology, Biosciences Institute, University College Cork, Cork, Ireland.

5.2 Blue/white selection

Blue/white screening was obtained for *E. coli* XL1-Blue on LB plate supplemented with antibiotic, 20 mg/ml of 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) and 0.4 M of isopropyl-ß-D-thiogalactopyranoside (IPTG).

5.3 PCR amplification and DNA fragment purification

Oligonucleotide primers used in this study are shown in Table 2. The PCR components for the amplification were done in 50 μl of PCR mixture and consist of 50 mM KCl, 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 200 μM of

each dNTP, 0.2 μM of each primer and 1 unit of *Taq* DNA polymerase. The PCR products derived from PCR or DNA fragment embedded in agarose gel were purified by using Gel/PCR DNA Fragments Extraction Kit (RBC, Bioscience Taiwan) according to the manufacturer's instruction. In briefly, one volume (one-hundred microliters) of PCR product was added with 5 volumes of DF buffer and mixed by vortexing. The sample mixture was applied into the DF column and centrifuged at 10,000 x *g* for 30 seconds. The flow-through was discarded. The DF column was added with 600 μl wash buffer and centrifuged at 10,000 x *g* for 30 sec. After centrifugation, the column matrix was dried by centrifugation for 2 minutes at 10,000 x *g*. The column matrix was added with 20-50 μl elution buffer at the center of the column and stand for 2 min until elution buffer or water was absorbed by the matrix. The purified DNA was eluted after centrifugation for 2 min at 10,000 x *g*. The eluted DNA was stored at -20°C until used.

For the DNA extraction from agarose gel, the agarose gel slice containing relevant DNA fragments up to 300 mg was excised and transferred into a microtube. The sample was added with 500 μl of DF buffer, mixed by vortexing, and was incubated at 55°C for 10-15 min until the gel slice had been completely dissolved. Eight hundred microliters of the sample mixture was applied into the DF column and centrifuged at 10,000 x *g* for 30 sec. After centrifugation, the flow through was discarded. The DF column was added with 600 μl wash buffer and centrifuged at 10,000 x *g* for 30 sec then discarded the flow-through. The column matrix was added with 20-50 μl elution buffer or water at the center of the column and stand for 2 min until elution buffer or water was absorbed by the matrix. The purified DNA was eluted after centrifugation for 2 minutes at 10,000 x *g*. The eluted DNA was stored at -20 °C until use. All steps in the purification were done at room temperature.

Table 2 Oligonucleotide primers used in this study

Oligonucleotides	Sequence (5'-3')
TT-F	ccg gtc tag ata gtc ttt gaa cct aa (*Xba*I)
TT-R	gaa cat atg gaa ttc aag ctc gcg tt (*Nde*I)
P919-F	gaa ttc tga agt gtg tcg tgt gg (*Eco*RI)
P919-R	cca tgg tgg tgt acc ttt tct tg (*Nco*I)
VP1-F	aaa tgc atg cgg gcg acc gcg tgg cc(*Nco*I)
VP1-R	ata taa gct tca ggg tgg tga tgg cg (*Xba*I)
XynF_F	gcc cat ggc cat gaa tta ttt acg 3'(*Nco*I)
XynF_R	cca tgg ttg aag atg gcg ttg aat (*Nco*I)
809_CWA_F	aat cta gaa cca ctg ccg tga cca (*Xba*I)
809_CWA_R	Aat aat cta gag tgc acg cgc tgg (*Xba*I)

5.4 DNA ligation

For cloning of PCR products into pGEM-T Easy vector (Promega, USA), the ligation was done according to the standard protocol as previously described [83]. The DNA ligation was done in 10 µl of final volume consisted of 1 µl of 10X ligation buffer, 1 µl of DNA T4 ligase, and the insert DNA and the vector in a molar ratio of 3:1 (insert:vector). The ligation reaction was incubated overnight at 16 °C. When needed, the appropriate amount of inserted DNA in the ligation reaction was provided as following equation:

$$\frac{\text{ng of vector} \times \text{kb size of insert DNA}}{\text{kb size of vector}} \times \text{insert : vector molar ratio} = \text{ng of inserted DNA}$$

5.5 Plasmid DNA isolation and purification

5.5.1 *E. coli* plasmids

Plasmids from *E. coli* were isolated by the alkaline lysis method as described by Green and Sambrook [83]. Briefly, a single colony of transformed *E.*

coli was grown in 2 ml of LB medium containing the ampicillin overnight at 37°C with shaking at 200 rpm. After overnight incubation, the bacterial cells were harvested by centrifugation at 14,000 x *g* for 5 min. The bacterial pellet was resuspended in 100 µl of 10 mg/ml lysozyme solution and 10 mg/ml of RNaseA, mixed by vortexing and stored on ice for 15 min. Two hundred microliters of freshly prepared NaOH/SDS solution was added to the bacterial suspension and mixed by inverting the tube rapidly for five times and stored on ice for 5 min. The lysed bacteria were then added with 150 µl of ice cold K solution, mixed thoroughly by inverting the tube for several times and stored on ice for 3-5 min. The supernatant was collected by centrifugation at 14,000 x *g* for 5 minutes, added with 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1). The upper phase was collected after centrifugation the solution at 13,000 x *g* for 10 min at room temperature. The plasmid DNA was precipitated out from the upper phase solution by adding 2 volumes of cold absolute ethanol and centrifuged at 14,000 x *g* for 10 min at room temperature. The DNA pellet was rinsed with 1 ml of 70 % ethanol followed by air-dried, and finally suspended in 50 µl of TE (pH 8.0).

5.5.2 Bifidobacteria plasmids

Plasmids of bifidobacteria were isolated and purified according to the previously described procedure [84] with slight modification. The bacteria were cultured under anaerobic conditions in 10 ml MRSC broth with or without antibiotics at 37 ^{o}C for 18 h. One ml of overnight culture was transferred into 1.5 ml microtube. The bacterial cells were then harvested by centrifugation at 14,000 x *g* for 5 min and washed with 1 ml of sterile distilled water. The bacterial cell pellet was suspended in 200 µl of STE buffer (pH 8.0) containing 30 mg/ml of lysozyme, 15 unit of mutanolysin and 50 mg/ml of RNase. After 2 h of incubation at 37 ^{o}C, the cell suspension was added with 400 µl of alkaline lysis solution and mixed by inversion. After 7 min of incubation, the cell suspension was added with 300 µl of 3 M sodium acetate (pH 4.8) and mixed by inversion. The supernatant of the mixture was collected by centrifugation at 14,000 x *g* for 10 min. A 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was

added into the supernatant and mixed by inversion. The upper phase was collected after centrifugation the solution at 13,000 x *g* for 10 min. The plasmid DNA was precipitated out from the upper phase solution by adding 2 volumes of cold absolute ethanol and centrifuged at 14,000 x *g* for 10 min at room temperature. Finally, the DNA pellet was rinsed with 1 ml of 70 % ethanol followed by air-dried and suspended in 50 µl of sterile distilled water or TE (pH 8.0).

All plasmid DNA used in this study are shown in Table 3.

Table 3 Plasmids utilized in this study.

Plasmid/vectors	Relevant properties	Source or reference
pGEM-T Easy	Ap^r, cloning vector	Promega
pLC13.9-Ldh-M2e:HBC-TT	pRCEID-LC13.9 containing gene expression cassette consists of Ldh-M2e-HBc	[85]
pGEM-TT	pGEM-T containing TT gene, Ap^r, cloning vector	This study
pGEM-P919	pGEM-T containing P919 constitutive promoter gene, Ap^r, cloning vector	This study
pUCIDT-BetA-VP1	pUCIDT containing gene expression BetA-VP1, Ap^r, cloning vector	Integrated DNA Technologies
pUCIDT-P919-VP1	pUCIDT containing gene expression P919-VP1, Ap^r, cloning vector	This study
pUCIDT-P919-VP1-TT	pUCIDT containing gene expression P919-VP1-TT, Ap^r, cloning vector	This study
pAM1 shuttle vector	*E. coli/Bifidobacterium* shuttle vector, Ap^r and Em^r	[86]
pAM1-P919-VP1-TT	Recombinant expression plasmid, Ap^r and Em^r	This study
pAM1-BetA-XynF-VP1-TT	Recombinant secretion-expression plasmid, Ap^r and Em^r	This study
pUCIDT-BetA-XynF-VP1-CWA-TT	pUCIDT containing gene expression BetA-XynF-VP1-CWA-TT, Ap^r, cloning vector	This study

Ap^r: Ampicillin resistance; Em^r: Erythromycin resistance

5.6 Preparation of bacterial competent cells

The centrifugation temperature used in the bacterial competent cell preparation was carried out at 4°C.

5.6.1 *E. coli* competent cells

A single colony of *E. coli* on LB agar plate was inoculated in 2 ml of LB medium and incubated overnight at 37°C with shaking at 250 rpm. The 1.5 ml of overnight culture was transferred to flask containing 150 ml of LB medium and further incubated until the OD_{600} reached 0.6. The bacterial cells was harvested by centrifugation at 12,000 x *g* for 15 min, washed twice in cold deionized water, the first wash with the volume of 150 ml and the second wash with the volume of 75 ml. After washing, the bacterial pellet was washed with 6 ml of ice-cold sterile 10% glycerol and the bacterial pellet was finally resuspended in 750 µl of ice-cold 10% glycerol. Seventy microliters of bacterial suspension was aliquoted in ice-cold sterile 1.5 ml microcentrifuge tube and stored at -70°C until use.

5.6.2 Bifidobacteria competent cells

Bifidobacterium competent cells were prepared according to the methods described by Alvarez-Martin [53]. In short, 100 ml of MRSC broth was inoculated with 8% culture of the overnight grown *B. pseudocatenulatum* M115 and incubated at 37°C for 3 to 4 h until the optical density at 600 nm reached 0.5 to 0.7. The cells were chilled for 20 min, washed twice in ice-cold sucrose-citrate buffer (0.5 M sucrose, 1 mM ammonium citrate [pH 5.8]), and resuspended in 100 µl of the same buffer. The competent cells can be used immediately or stored at –80˚C until use.

5.7 Electroporation

5.7.1 *E. coli*

The frozen competent *E. coli* cells were thawed on ice, added with one microliter of a standard ligation reaction and mixed gently on ice. The mixture was transferred to ice-chilled electroporation cuvette and placed the cuvette into

electroporation apparatus. The electroporation was done with an electric pulse of 1.8 kV and constant time of 4-5 milliseconds. After electroporation, the mixture was quickly transferred to 1 ml of SOC medium and incubated with gentle shaking (150 rpm) for 1 h at 37°C. After incubation, the transformed cells were spread onto LB agar containing appropriate antibiotic, and incubated at 37°C for 24 h.

5.7.2 Bifidobacteria

For electrotransformation [53], the cell suspension was stored on ice for 20 min before use. One microgram of plasmid DNA was mixed with 50 µl of ice cold competent cells and filled in ice-chilled electroporation cuvette. Electroporation was performed at 25 µF, 200, and 10 kV. The cells were added in 950 µl of RCM broth and incubated for 2 h and 30 min. After incubation, plated onto MRSC agar medium supplemented with the 2.5 µg/ml erythromycin and incubated for 2 to 3 days at 37°C under anaerobic condition.

5.8 Construction of expression plasmids for VP1 expression

5.8.1 VP1 codon optimization

The VP1 gene of enterovirus 71 (accession no. JF738000.1) was retrieved from NCBI database and its codon were optimized for efficient expression in *Bifidobacterium* species based on the codon usage Database preferentially used *Bifidobacterium breve* UCC2003 (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=326426) which is the primary expression host we intent to use. The codon-optimized VP1 gene was synthesized by an Integrated DNA Technologies (IDT) with 5' *Nco*I (CCATGG) and 3'*Xba*I (TCTAGA) flanking site. In addition, the BetA prompter was added upstream of VP1 gene with 5' *Eco*RI (GAATTC) and 3' *Nco*I (CCATGG) flanking site. The BetA prompter is the cholic acid-induced promoter derived from *Bifidobacterium longum* NCC2705 [59]. The synthesized BetA-VP1 gene fragment was supplied as the inserted gene fragment in pUC19 plasmid and named pUCIDT-BetA-VP1.

5.8.2 Construction of recombinant expression plasmid containing VP1

Primarily, the BetA-VP1 gene fragment was subcloned into pAM1,the *E. coli/Bifidobacterium* shuttle vector, with the addition of the transcriptional terminator (TT) to generate recombinant expression plasmid containing VP1 designated as pAM1-BetA-VP1-TT. However, attempt to introduce this recombinant plasmid into *B. breve* UCC2003 was unsuccessful. For this reason, the expression host was changed from *B. breve* UCC2003 to *B. pseudocatenulatum* M115. It was found that *B. pseudocatenulatum* M115 was successfully electrotransformed with pAM1-BetA-VP1-TT. However, attempt to induce the VP1 expression with cholic acid in this host was unsuccessful. Thus, in this study the promoter of this recombinant expression plasmid was changed from BetA inducible promoter to P919 constitutive promoter [58] resulting a new recombinant expression plasmid containing VP1 designated as pAM1-P919-VP1-TT.

For construction of pAM1-P919-VP1, P919 promoter with the length of 206 bp was amplified from ProSP (gBlocks 429 base pairs) and cloned into pGEM-T Easy vector resulting in pGEM-P919. ProSP is synthetic gene fragment consist of P919 promoter sequence of *B. bifidum* BGN4 and xynF signal peptide gene of *B. longum*. P919 promoter was reamplified from pGEM-P919 with 5'*Eco*RI and 3'*Nco*I. The P919 amplified product was cut with *Eco*RI and *Nco*I, subjected to agarose gel electrophoresis and purified out of gel with Gel/PCR DNA Fragments Extraction Kit as detailed in section 2.3. The PCR condition and the specific primer pairs are detail in Table 6 (Appendix). The purified P919 was ligated to *Eco*RI/*Nco*I digested pUCIDT-BetA-VP1 and electrotransformed into *E.coli* XL1-blue. The pUCIDT-P919-VP1 derived from transformant *E. coli* XL1-blue was verified by *Eco*RI/*Nco*I-digestion and sequencing.

The pUCIDT-P919-VP1 was further used to construct pUCIDT-P919-VP1-TT. The terminal terminator with the length of 322 bp was amplified from

pLC13.9-Ldh-M2e:HBc-TT and cloned into pGEM-TT Easy resulting in pGEM-TT. Similar to the process to insert of P919 into the construct above, the TT amplified product with 5'*Xba*I and 3'*Nde*I flanking was inserted downstream of VP1 of pUCIDT-P919-VP1 to generate pUCIDT-P919-VP1-TT.

To construct pAM1-P919-VP1-TT, the P919-VP1-TT gene fragment derived from *Eco*RI-digested pUCIDT-P919-VP1-TT was purified from gel and ligated to *Eco*RI-digested pAM1 and electrotransformed in *E. coli* XL1-blue. The construct derived from the transformant *E. coli* XL1-blue was further verified by *Eco*RI digestion and sequencing. The correct construct was further used to electrotransformed in *B. pseudocatenulatum* M115 and the transformant *B. pseudocatenulatum* M115 was used to analyse the VP1 expression.

5.9 Expression of VP1in *Bifidobacterium pseudocatenulatum* M115

The procedure used for inducing the expression of VP1protein was performed according to the previous study [57]. A single colony of the recombinant *B. pseudocatenulatum* M115 was inoculated in 10 ml of MRSC medium supplemented with 2.5 µg/ml of erythromycin and statically incubated at 37°C for 18 h. The bacterial cells were harvested by centrifugation and then transferred into new 100 ml MRSC medium supplemented with 2.5 µg/ml of erythromycin and were grown at 37°C until the mid-exponential-phase (optical density at 600 nm reached 0.8 at 7-9 h) of the culture is obtained [58]. After incubation, the bacterial cells were washed twice with phosphate buffer saline (PBS, pH7.0) by centrifugation 4,500 rpm, 15 min at 4˚C, and suspended in lysis buffer (0.5 M Na_2HPO_4, 5 M NaCl, 1 M imidazole, 100 mg/ml lysozyme, 1X protease inhibitor cocktail (Amresco, USA), 1 M dithiothreitol). The bacterial cells were disrupted in eppendorf tube with glass beads for three times of 30 s at full speed, with cooling on ice for 1 minutes. The bacterial lysates were harvested by centrifugation for 15 min at 14,000 rpm and 4°C to remove beads and bacterial debris [18]. The protein concentrations in the lysates were measured by Bradford protein assay.

5.10 Analysis of the expression proteins using SDS-PAGE and western blot

The expressed proteins were subjected to SDS-PAGE with 5% stacking gel and 12% separating gel. The plates were cleaned with 95% ethanol and dried before used. The protein samples were mixed with 2xSDS loading dye buffer and boiled for 5 min for protein denaturation. The denatured protein samples and the protein standard marker (PageRulerTM Prestained Protein Ladder, Fermentas, USA) or Chromatein Prestained (Vivantis, USA) was subjected to SDS-PAGE at 100 V for 2 h. After electrophoresis, the proteins in gel can either be visualized by staining with Coomassie brilliant blue R-250 or further analyzed by western blot analysis.

For western blot analysis, proteins in gel were transferred to a positively charged membrane by semi-dry electroblotting. For the semi-dry electroblotting method, stacks of filter paper (Bio-Rad, USA) and nirocellulose membrane (Bio-Rad, USA) were prewetted in semi-dry transfer buffer. Stacks of prewetted filter papers were placed at the cathode and the anode. The gel and the membrane were sandwiched between one stack of filter paper. The gel was placed near the cathode and the membrane was placed near the anode. The proteins in the gel were transferred onto the membrane by applying an electrical field at 25 V for 60 min.

For immunodetection, the membrane was washed twice with TBS buffer for 10 min each and was incubated for 1 h in blocking buffer. After blocking, the membrane was washed once in TBS-Tween/Triton buffer for 10 min. The membrane was then incubated overnight with mouse anti-VP1 monoclonal antibody (Abnova, Taiwan) with the dilution of 1/1,000 at room temperature. After incubation, the membrane was washed three times in TBS-Tween/Triton buffer for 10 min and incubated with goat anti- mouse IgG monoclonal conjugated HRP (Santa Cruz Biotechnology, USA) with the dilution of 1/10,000 at room temperature for 1 h. After incubation, the membrane was washed 4 times with TBS-Tween/Triton buffer and once with TBS each for 10 min. The detection signal was developed by incubation with 1 ml

of SuperSignal West Substrate Working Solution (SuperSignal® Chemiluminescent Substrate, Thermoscience, USA) for 5 min and the signal was recorded by using the digital camera (Image Quant TM LAS 4000, Sweden)

5.11 Plasmid stability assay

The segregational stability of the construct pAM1-P919-VP1-TT and pAM1-P919-VP1-TT expression vector were studied in *B. pseudocatenulatum* M115. The transformant with each construct was grown in MRSC broth without erythromycin for approximately 160 generations. Every 20 generations, an aliquot of the culture was removed, diluted, and plated onto antibiotic-free MRSC medium. One hundred colonies were selected and then replicated on MRS agar with and without erythromycin. For segregational stability, colonies grown on MRSC agar with and without antibiotics were counted and calculated as follow: $(N_A/N_{NA}) \times 100$, where N_A and N_{NA} is number of colonies appearing on MRSC plate with and without erythromycin, respectively.

5.12 Nucleotide sequence accession number

The complete nucleotide sequence of synthetic VP1 was deposited in the GenBank database under accession number KY115200.

6. Results

6.1 Codon optimization of VP1 gene for expression in Bifidobacterium.

To adjust the codon usage that frequently used by *Bifidobacterium breve*, the native nucleotide sequence of viral capsid protein1 (VP1) was analyzed by comparing the codon usage preference of *Bifidobacterium breve* UCC2003. It was found that VP1 gene contains some codons that appear to be rare codons in *Bifidobacterium breve* as they are rarely found in their genome at less than 0.1 % of whole genome.

These codons consist of 5 ATA coding for isoleucine, 3 TTA coding for leucine, and 3 CGG coding for arginine. For optimization, all codons (rare codons and other) were replaced with the codons those highly used by *Bifidobacterium breve*. For example, the CGT, CGC, CGA, CGG (rare codon), AGA, AGG coded for arginine were replaced with CGC, the highest codon usage in *B. breve*. The result of codon optimization of VP1 gene is summarized in Table4.

For the expression of VP1 in *B. breve* UCC2003, the bile-inducible gene-derived promoter or betA of *Bifidobacterium longum* subsp. *longum* NCC2705 was used (2). The 469 bp betA promoter was retrieved from the NCBI database at http://www.ncbi.nlm.nih.gov under an accession number AE014295.3. For the facility of cloning, *Eco*RI sequence (GAATTC) and *Nco*I (CCATGG) was added to the 5'- and 3'-end, respectively, of betA.

Finally, betA promoter was placed at 5'-end of VP1 to obtained the fusion gene betA:VP1 gene with the length of 1,403 bases (Fig 2). This fusion gene was synthesized and cloned into pUC19 cloning vector, resulting vector named as pUCIDT-AMP, which is then named as pUCIDT-BetA-VP1 (Fig 3). This vector was commercially obtained from Integrated DNA technologies, Co. Ltd., USA. The pUCIDT-BetA-VP1 was digested with different restriction enzymes to be sure that the vector has correctly physical and genetic map. After digestion, it was proven that the pUCIDT-BetA-VP1 vector had a fusion gene with the corrected orientation (Fig 4). The pUCIDT-BetA-VP1

vector was electrotransformed into *E. coli*. The bacterial stock frozen was prepared and kept at -80 °C until used.

Table 4 Native- and codon-optimized VP1 gene. The number of each codon found the native VP1 sequence was showed in parenthesis.

Amino acid	Codon Native*	Optimized	Amino acid	Codon Native*	Optimized
Arg (R)	CGT (2), CGC (2), CGA (1), **CGG (3)**, AGA (4), AGG (4)	CGC	Ser (S)	AGC (6), AGT (6), TCA (6), TCC (4), TCG (3), TCT (1)	TCC
Gly (G)	GGT (5), GGC (4), GGA (3), GGG (7)	GGC	Val (V)	GTA (3), GTC (6), GTG (6), GTT (5)	GTG
Leu (L)	CTA (1), CTC (9), CTG (1), CTT (1), **TTA (3)**, TTG (3)	CTG	Ala (A)	GCA (8), GCC (7), GCG (7), GCT (5)	GCC
Pro (P)	CCA (10), CCC (7), CCG (2), CCT (4)	CCG	Ile (I)	**ATA (5)**, ATC (2), ATT (5)	ATC
Cys (C)	TGC (0), TGT (3)	TGC	Gln (Q)	CAA (11),CAG (1)	CAG
Thr (T)	ACA (6), ACC (11), ACG (4), ACT (4)	ACC	Asn (N)	AAC (8), AAT (6)	AAC
Lys (K)	AAA (6), AAG (5)	AAG	Asp (D)	GAC (8), GAT (6)	GAC
Phe (F)	TTG (6), TTF (7)	TTC	His (H)	CAC (3), CAT (1)	CAC
Tyr (Y)	TAC (4),TAT (8)	TAC	Glu (E)	GAA (3), GAG (11)	GAG
Met (M)	ATG (10)	ATG	Trp (W)	TGG (4)	TGG

*In parentheses, the number of each codon found in the native VP1 sequence.

*Rare codons found in the native VP1 gene are shown in bold.

LOCUS: BetAVP1 **Size:** 1403 bp **DNA type:** LINEAR
ORGANISM: *Bifidobacterium breve* (bases 1 to 475), Enterovirus 71 (bases 476 to 1377)
AUTHORS: Marutpong Panya, Baltasar Mayo, Viraphong Lulitanond, and Thapanee Thinbanmai
File: created by SciEd Central, Scientific & Educational Software
BASE COUNT 264 a 494 c 381 g 264 t
ORIGIN

```
      1 GAATTCTATG CGGTAAGCCC AATCCTTTTG TGAAGATTTT GTGTATTGTC AGGCATGCGT
     61 GGGTTTCACC GTCGATTTGA ATGATGATAA GCTTCTCGAT GTCTGCCGTA CGAAGACCCC
    121 ATCAAGGAGA TGCCATGAAC CTGTATCGCT ACGCGATGTT CATCGCACGC GCCTGCCGTG
    181 GGTCGTTCGT CGGCAATGTT CTGTTTGCCA ATCCACGAAT GGCCCTATTC TCGATGCTGG
    241 TGTAGCGGGG ACACGCCTTA TGCCGGGATG TACCGATGGC CCATCCGCAG TCGACGTCGC
    301 ATGAATCGCT CACCATAGCC ATCAACTTAC GCAAAGCCGC GAATCGTTTG CCGTATATGA
    361 ATGCGGAACG GATGGCGGCT TTTCCTTTAC GTCCGGACTC ATCGGTGTCC TCGTGTCTTG
    421 ACACAAGCAT GGGGCCGGAT CGCCACACAT GACGTTACCA ACAGAAGGAA ATGCTCCATG
    481 GGCGACCGCG TGGCCGACGT GATCGAGTCC TCCATCGGCG ACTCCGTGTC CCGCGCCCTG
    541 ACCCAGGCCC TGCCGGCCCC GACCGGCCAG AACACCCAGG TGTCCTCCCA CCGCCTGGAC
    601 ACCGGCAAGG TGCCGGCCCT GCAGGCCGCC GAGATCGGCG CCTCCTCCAA CGCCTCCGAC
    661 GAGTCCATGA TCGAGACCCG CTGCGTGCTG AACTCCCACT CCACCGCCGA GACCACCCTG
    721 GACTCCTTCT TCTCCCGCGC CGGCCTGGTG GGCGAGATCG ACCTGCCGCT GGAGGGCACC
    781 ACCAACCCGA ACGGCTACGC CAACTGGGAC ATCGACATCA CCGGCTACGC CCAGATGCGC
    841 CGCAAGGTGG AGCTGTTCAC CTACATGCGC TTCGACGCCG AGTTCACCTT CGTGGCCTGC
    901 ACCCCGACCG GCGAGGTGGT GCCGCAGCTG CTGCAGTACA TGTTCGTGCC GCCGGGCGCC
    961 CCGAAGCCGG ACTCCCGCGA GTCCCTGGCC TGGCAGACCG CCACCAACCC GTCCGTGTTC
   1021 GTGAAGCTGT CCGACCCGCC GGCCCAGGTG TCCGTGCCGT TCATGTCCCC GGCCTCCGCC
   1081 TACCAGTGGT TCTACGACGG CTACCCGACC TTCGGCGAGC ACAAGCAGGA GAAGGACCTG
   1141 GAGTACGGCG CCTGCCCGAA CAACATGATG GGCACCTTCT CCGTGCGCAC CGTGGGCACC
   1201 TCCAAGTCCA AGTACCCGCT GGTGATCCGC ATCTACATGC GCATGAAGCA CGTGCGCGCC
   1261 TGGATCCCGC GCCCGATGCG CAACCAGAAC TACCTGTTCA AGGCCAACCC GAACTACGCC
   1321 GGCAACTCCA TCAAGCCGAC CGGCGCCTCC CGCACCGCCA TCACCACCCT GTCTAGAGGT
   1381 ACCCTCGAGT TCATATGACT AGT
//
```

Figure 2 Nucleotide sequence of synthetic betA:VP1 gene.

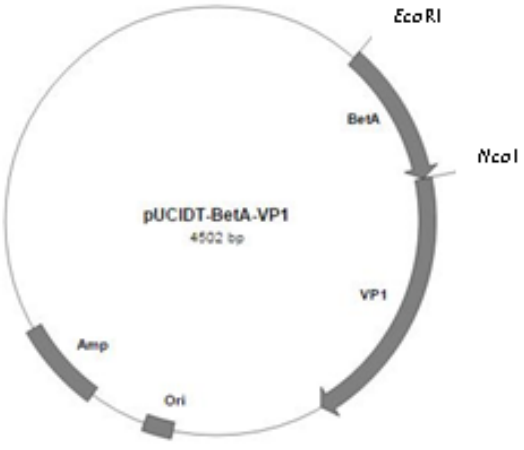


Figure 3 Physical and genetic map of the plasmid pUCIDT-BetA-VP1.

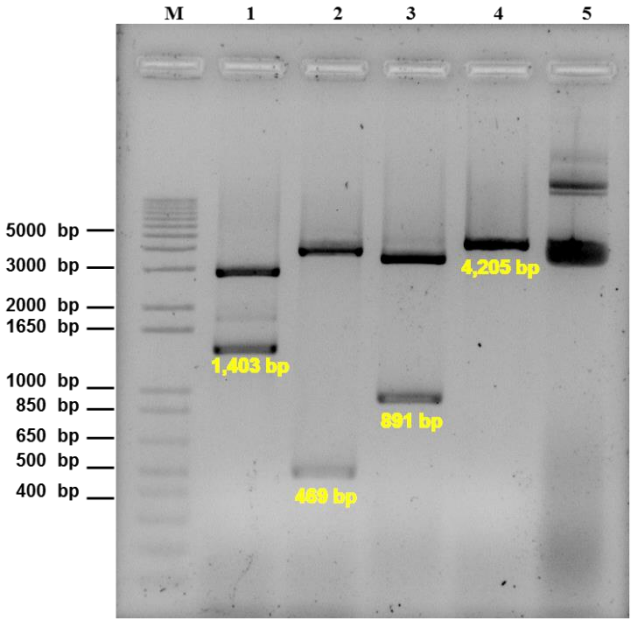


Figure 4. SYBR Green staining gel of restriction enzyme-digested pCMP_1. Land M: Marker 1 kb plus DNA Ladder. Land 1: pUCIDT-BetA-VP1 digested by *Eco*RI and *Xba*I. Land 2: pUCIDT-BetA-VP1 digested by *Eco*RI and *Nco*I. Land 3: pUCIDT-BetA-VP1 digested by *Nco*I and *Xba*I. Land 4: pUCIDT-BetA-VP1 digested by *Nco*I. Land 5: Undigested pUCIDT-BetA-VP1 plasmid.

6.2 Construction of an expression vector under the control of betA inducible promoter

The 332 bp of transcription terminator (TT) was amplified from the pLC13.9-Ldh-M2e:HBC-TT (Our laboratory research) by PCR using primers (Table 2) and amplification condition (Table 6). The amplified DNA (Fig. 5) was directly cloned into pGEM-T Easy vector to produce recombinant plasmid named pGEM-TT. The 332-bp TT DNA fragment was then removed from pGEM-TT by *Xba*I/*Nde*I digestion and was cloned into the *Xba*I/*Nde*I-digested pUCIDT-BetA-VP1, resulting in pBetA-VP1-TT. The construct was verified by restriction enzyme digestion and DNA sequencing. According to the result of restriction analysis and DNA sequencing of the construct pBetA-VP1-TT, it was demonstrated that the expression cassette which contains the consecutive gene of BetA promoter, VP1, and TT gene, was successfully generated (Fig. 6 and Fig. 7).

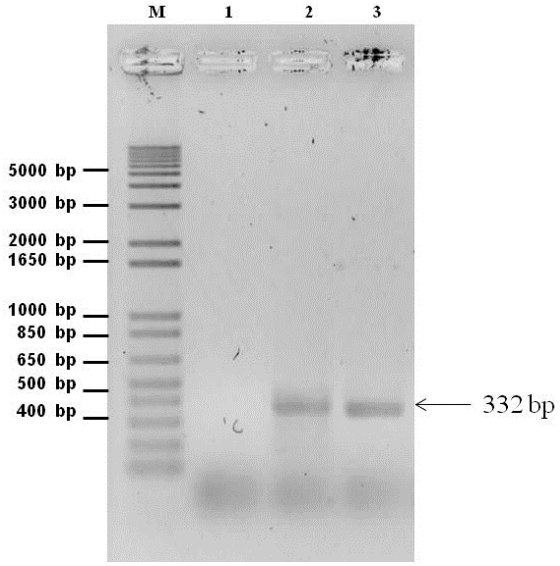


Figure 5. SYBR Green staining gel of amplified transcriptional terminator (TT) from pLC13.9-Ldh-M2e:HBC-TT. Land M: Marker 1 kb plus DNA Ladder. Land 1: negative control. Land 2 and 3: amplified TT from pLC13.9-Ldh-M2e:HBC-TT plasmid.

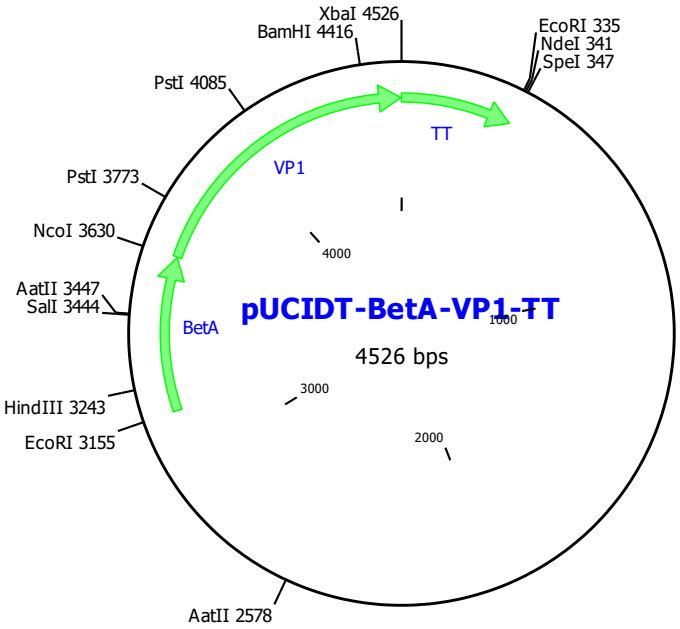


Figure 6 Physical and genetic map of the plasmid pUCIDT-BetA-VP1-TT.

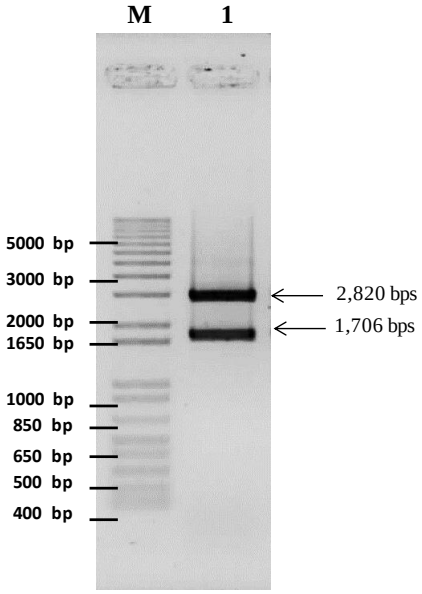


Figure 7 SYBR Green staining gel of restriction enzyme-digested pUCIDT-BetA-VP1-TT. Land M: Marker 1 kb plus DNA Ladder. Land 1: *Eco*RI-digested pUCIDT-BetA-VP1-TT.

The expression cassette betA:VP1:TT gene was obtained from the pUCIDT-BetA-VP1-TT. The construct pUCIDT-BetA-VP1-TT was digested with *Eco*RI restriction enzyme. The betA:VP1:TT gene fragment was cloned into the *EcoR*I-digested pAM1, *E.coli*/bifidobacterium shuttle vector, resulting the new expression vector named pAM1-BetA-VP1-TT (Fig 8.).

To verify the new expression vector, pAM1-BetA-VP1-TT, four transformants *E. coli* were selected and used for plasmid isolation and restriction enzymes digestion. The isolated plasmid fro4m each transformant was digested with *Eco*RI and *Pst*I. Digestion of all constructs except clone1 generated two DNA fragments as follow; 1706 bp and 6205 bp (Fig 9A). Digestion of all constructs with *Pst*I generated three DNA fragments as follow; 312 bp, 3423 bp. and 4176 bp (fig 9B). Restriction analysis result indicated that the new expression vector designated as pAM1-BetA-VP1-TT was successfully constructed. Finally, DNA sequencing result found that all plasmid constructs had the corrected sequence and orientation (data not shown). For further study, clone2 was electro-transformed into *E. coli*, *B. breve* UCC2003 and *B. pseudocatenulatum* M115.

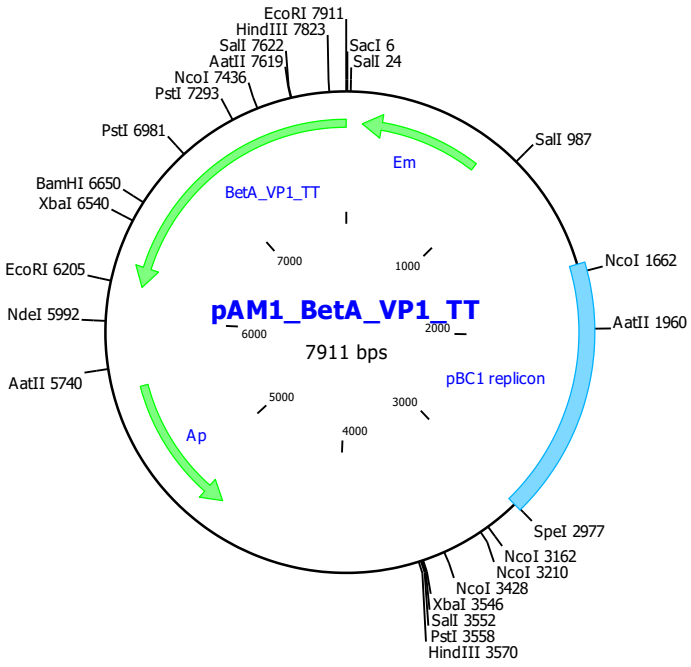
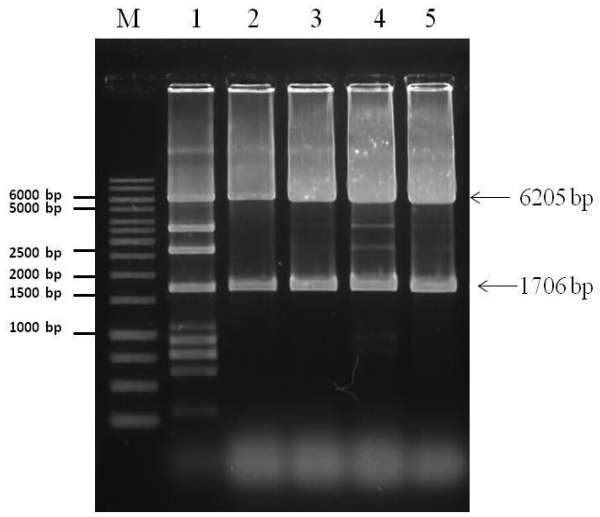


Figure 8 Physical and genetic map of the plasmid pAM1-BetA-VP1-TT.

9A.



9B.

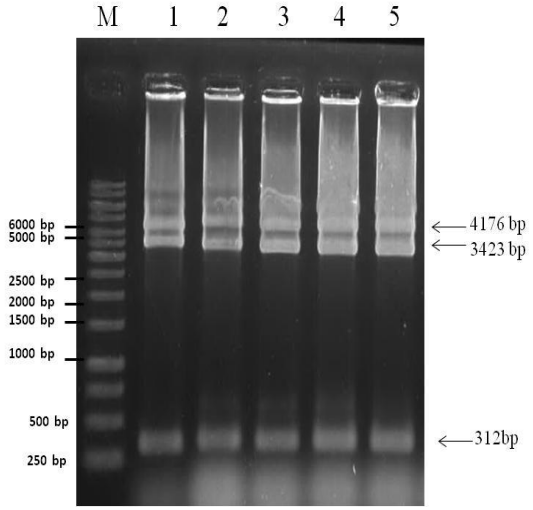


Figure 9 SYBR Green staining gel of restriction enzyme-digested pAM1-BetA-VP1-TT. Lane M: Marker 1 kb plus DNA Ladder. 9A, Lane 1, 2, 3, 4, and 5: *Eco*RI-digested clone1, cloned2, clone3, clone4, and clone5, respectively**.** 9B, Lane 1, 2, 3, 4, and 5: *Pst*I-digested clone1, cloned2, clone3, clone4, and clone5, respectively.

6.3 Construction of an expression vector under the control of P919 constitutive promoter

Figure10 shows the construction of pAM1-P919-VP1-TT. From the beginning of this study, we found the limitation for using restriction enzyme, the P919 promoter could not be cloned directly into the pAM1-BetA-VP1-TT vector instead of betA promoter. Therefore, several cloning steps were performed. The P919 promoter with the length of 206 bp was amplified from ProSP, a 429 bp synthetic gene consisted of P919 promoter sequence of *B. bifidum* BGN4 and xynF signal peptide gene of *B. longum*. (gBlocks, Singapore). The amplified product was cloned into pGEM-T Easy vector resulting in pGEM-P919. P919 promoter was further amplified from pGEM-P919. The P919 amplified product was digested with *Eco*RI and *Nco*I, subjected to agarose gel electrophoresis and was purified out of gel with Gel/PCR DNA Fragments Extraction Kit. The purified P919 fragments were ligated to *Eco*RI/*Nco*I digested pUCIDT-BetA-VP1 and electrotransformed into *E.coli* XL1-blue. The pUCIDT-P919-VP1 derived from transformant *E. coli* XL1-blue was verified by *Eco*RI/*Nco*I-digestion and followed by sequencing.

The pUCIDT-P919-VP1 was further used to construct pUCIDT-P919-VP1-TT. The terminal terminator (TT) with the length of 322 bp was amplified from pGEM-TT. The terminal terminator with 5'*Xba*I and 3'*Nde*I flanking was inserted downstream of VP1 in the vector pUCIDT-P919-VP1 to generate pUCIDT-P919-VP1-TT.

To construct pAM1-P919-VP1-TT, the P919-VP1-TT gene fragment derived from *Eco*RI-digested pUCIDT-P919-VP1-TT was purified from agarose gel and ligated to *Eco*RI-digested pAM1, to generate an expression vector pAM1-P919-VP1-TT. The vector was then electrotransformed in *E. coli* XL1-blue. The construct derived from the transformant *E. coli* XL1-blue was further verified by *Eco*RI digestion and sequencing. The correct construct was further used to electrotransformed in *Bifidobacterium breve* UCC2003 and *B. pseudocatenulatum* M115 and the transformant was used to analyst the VP1 expression.

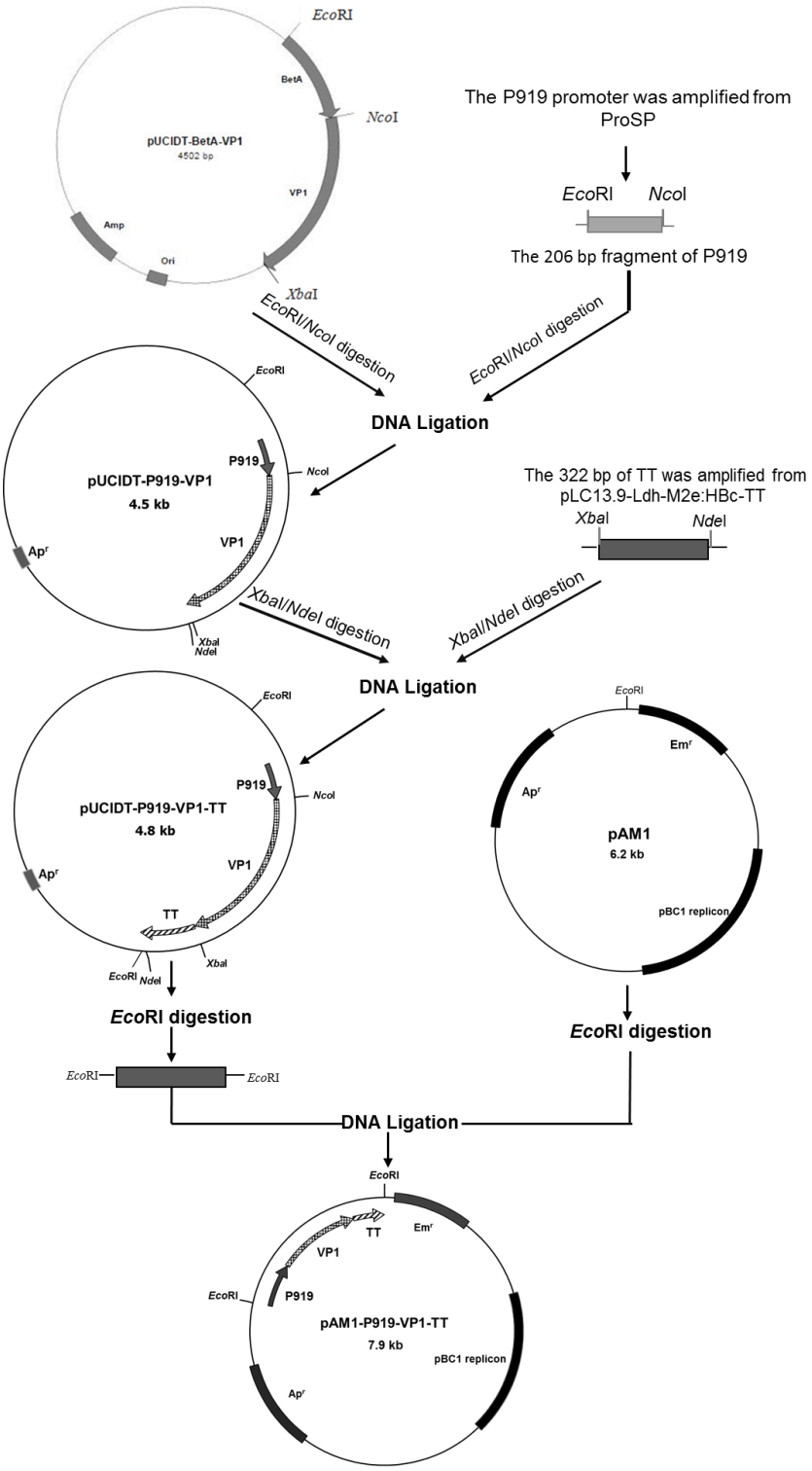


Figure 10 Schematic diagrams of construction of the recombinant pAM1-P919- VP1-TT. The Ap^r, Em^r, ori, and repB indicate ampicillin resistant gene, erythromycin resistant gene, origin of replication and replicon B, respectively.

After the construction of pUCIDT-P919-VP1 with the length of 3,942 bp, this construct was verified by *Eco*RI/*Nco*I digestion as shown in figure 11. *Eco*RI/*Nco*I digested pUCIDT-P919-VP1 generate two DNA fragment with the length of 3,736 and 206 bp. For pUCIDT-P919-VP1-TT with the length of 4,264 bp, digestion this construct with *Xba*I/*Nde*I generated two DNA fragment with the length of 3,942 and 322 bp as shown in figure 12 while digestion the construct with *Eco*RI generates 2,840 and 1,424 bp-DNA fragments as shown in figure 13. After sequencing verification, the sequence analysis using Bioedit shown that the orientation of P919-VP1 and TT was corrected as shown in figure 14. The final recombinant expression plasmid carrying VP1, pAM1-P919-VP1-TT, was verified by *Eco*RI digestion. As shown in figure 15, the *Eco*RI-digested construct generates two DNA fragments with the length of 6,025 and 1,424 bp.

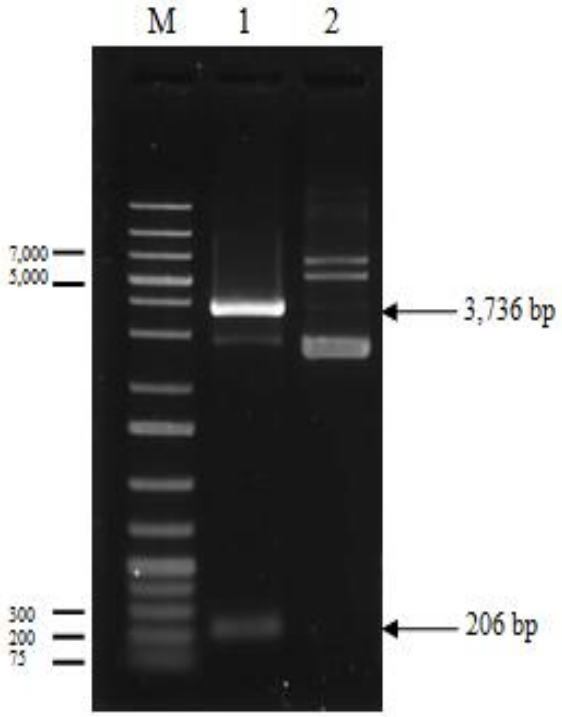


Figure 11 SYBR Gold staining gel *Eco*RI and *Nco*I-digested fragment recombinant plasmids pUCIDT-P919-VP1. Lane M= 1kb plus DNA ladder, Lane 1= *Eco*RI and *Nco*I digested recombinant plasmids and Lane 2= undigested recombinant plasmids pUCIDT-P919-VP1.

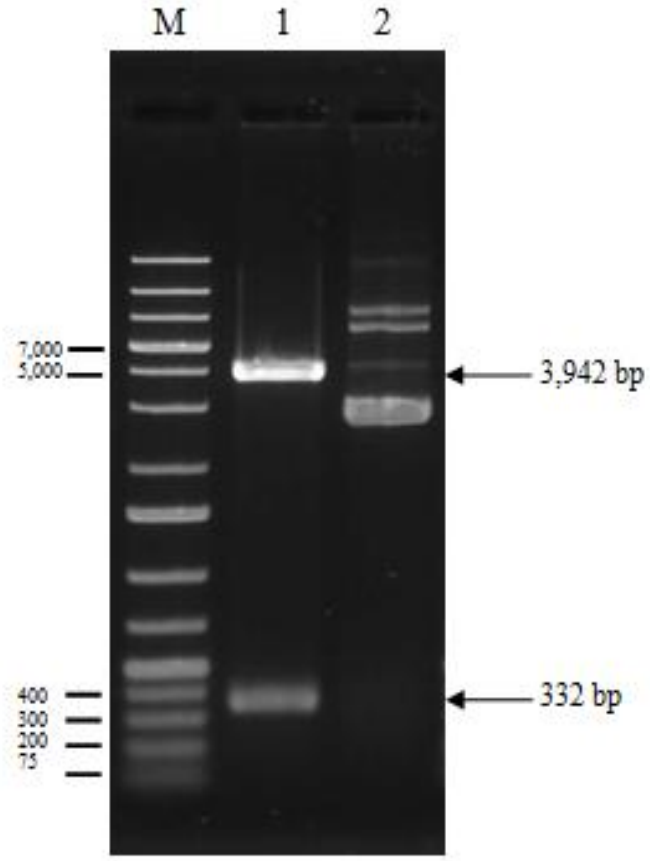


Figure 12 SYBR Gold staining gel of *Xba*I and *Nde*I-digested pUCIDT-P919-VP1-TT. Lane M= 1kb plus DNA ladder, Lane 1= *Xba*I and *Nde*I digested plasmid and Lane 2= undigested plasmid.

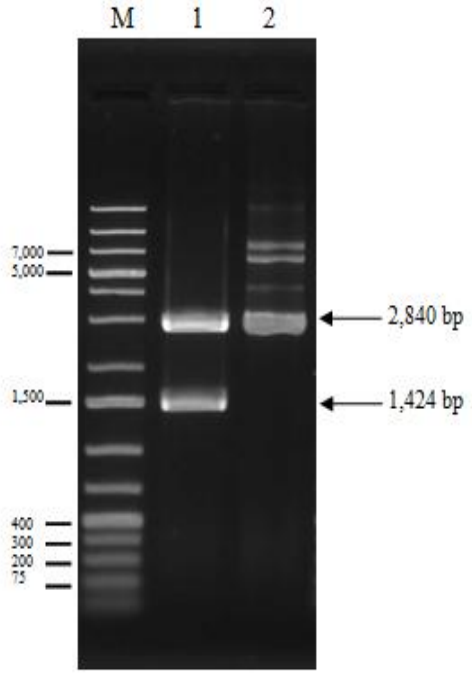


Figure 13 SYBR Gold staining gel of *Eco*RI-digested pUCIDT-P919-VP1-TT. Lane M= 1kb plus DNA ladder, Lane 1= *Eco*RI digested plasmid and Lane 2= undigested plasmid.

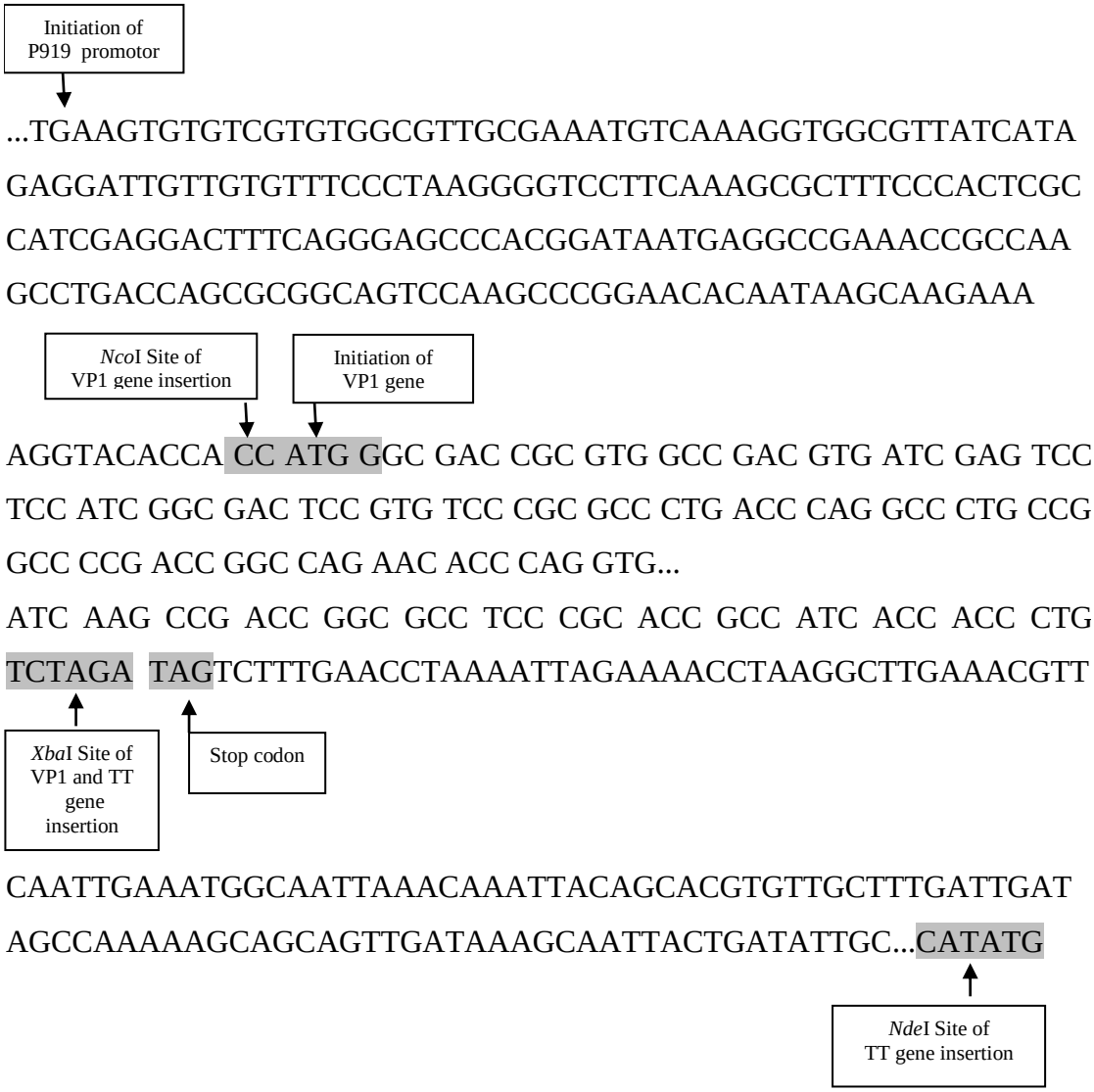


Figure 14 Sequence analysis of pUCIDT-P919-VP1-TT by Bioedit. The sequence shows the correct orientation of P919 promoter and start codon. The sequence of VP1 gene was in-frame. Stop codon of TT, TAG was presented.

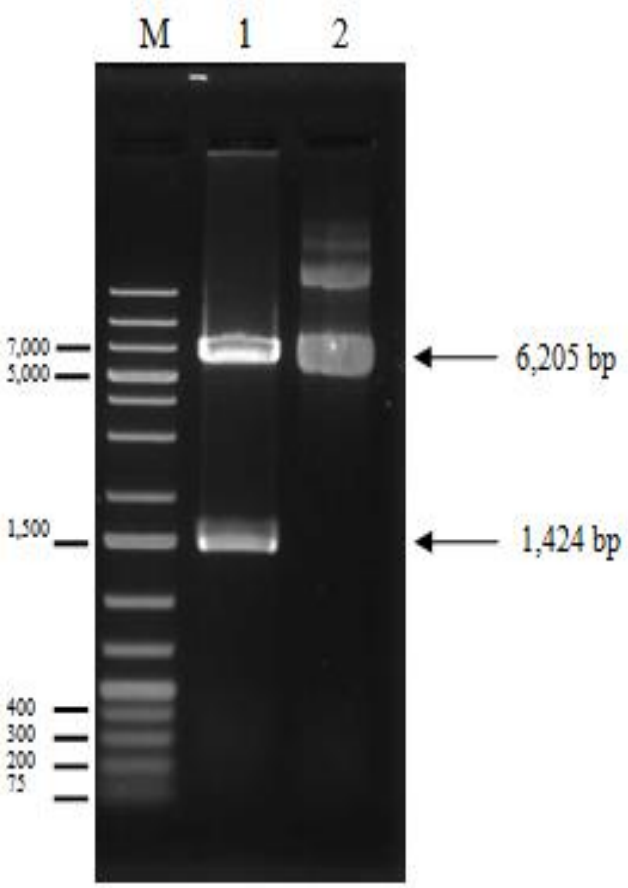


Figure 15 *Eco*RI-digested fragment recombinant expression plasmid pAM1-P919-VP1-TT. Lane M= 1kb plus DNA ladder, Lane 1= *Eco*RI digested plasmid and Lane 2= undigested plasmid.

6.4 Construction of recombinant expression plasmid pQE31-VP1

To construct the expression plasmid for controlling the expression of VP1 in *E. coli* which can be further used as positive control in western blot analysis. The DNA fragment of VP1 with the length of 891 bp derived from *Nco*I/*Xba*I-digested pUCIDT-BetA-VP1 was inserted into *Nco*I/*Xba*I-digested pQE31 vector to produce recombinant pQE31-VP1 (Figure 16). This recombinant plasmid was then transformed into *E. coli* XL1-blue. The construct pQE31-VP1 was verified by *Bam*HI digestion and sequencing.

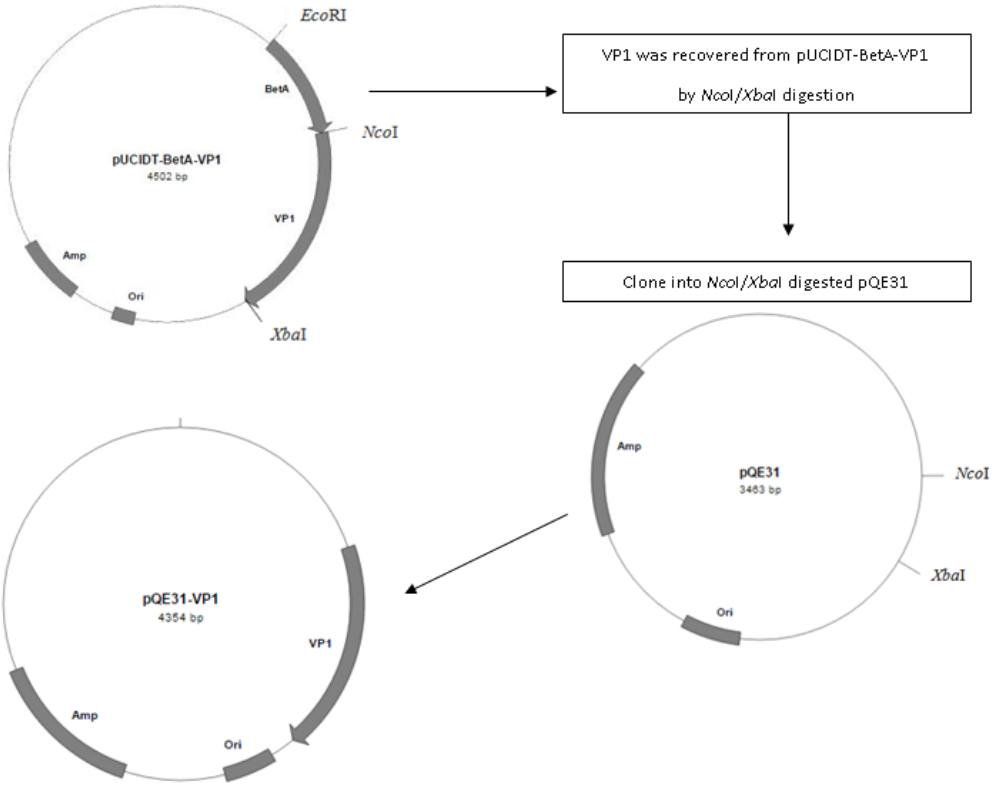


Figure 16 Schematic diagram for the construction of pQE31-VP1. The Amp and Ori indicate ampicillin resistant gene and origin of replication, respectively.

The recombinant pQE31-VP1 was constructed and was introduced into *E.coli* XL1-blue. The positive transformants were identified by *Bam*HI digestion. *Bam*HI-digested pQE31-VP1 generates two DNA fragments with the length of 792 and 3562

bp. (Fig.17, lane 1). Further verification of this constructs was done by sequencing. The result showed the nucleotide sequences and orientations of insert was corrected (data not shown).

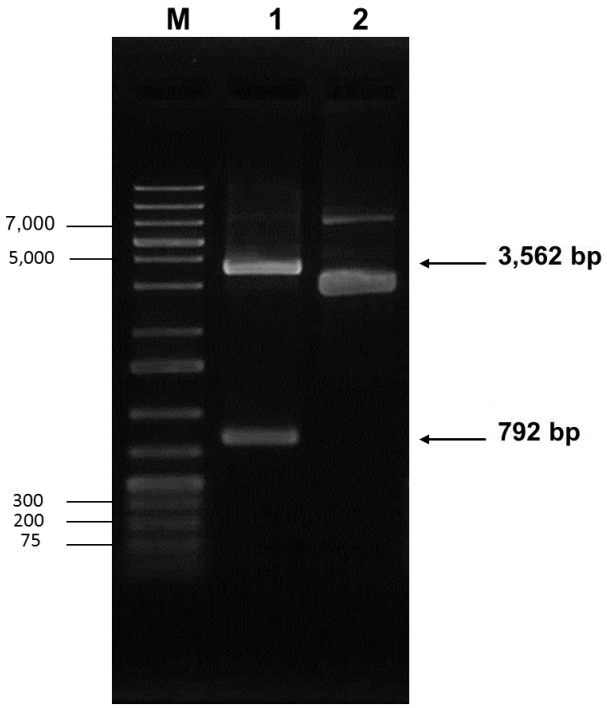


Figure 17 SYBR Gold staining gel of *Bam*HI-digested pQE31-VP1. Lane M: 1kb plus DNA ladder, lane 1: *Bam*HI-digested pQE31-VP1and lane 2: undigested pQE31-VP1.

6.5 Transformation of recombinant expression plasmid into *Bifidobacterium breve* UCC2003 and *Bifidobacterium pseudocatenulatum* M115

Two recombinant expression plasmids, pAM-BetA-VP1-TT and pAM-P919-VP1-TT, was individually transformed into *B. breve* UCC2003 and *B. pseudocatenulatum* M115. The condition used to prepare the electrotransformation is shown in Table5. Primarily, the recombinant expression plasmid containing VP1 under the control of inducible promoter BetA, designated as pAM1-BetA-VP1-TT, was unsuccessful to introduce into those two strains, although several attempts with different electroporation conditions were performed. For this reason, the expression host was changed from *B. breve* UCC2003 to *B. pseudocatenulatum* M115, a human isolate that has been proven

as probiotic for human use (IPLA Laboratory collection). After transformation it was found that *B. pseudocatenulatum* M115 was successfully electrotransformed with pAM1-BetA-VP1-TT as shown in Table5. In case of pAM-P919-VP1-TT, the transformation efficiency showed the similar result with that was obtained from pAM1-BetA-VP1-TT. This result suggested that the constructs were not function in *B. breve* UCC2003. For this reason, we selected *B. pseudocatenulatum* M115 as bacterial host for further construction.

Table 5 Transformation efficiency of the recombinant expression plasmids in bifidobacterium.

Electroporation condition	Transformation efficiency (number of transformant/μg)					
	pAM1-BetA-VP1-TT			pAM-P919-VP1-TT		
	E.coli	*B. breve* UCC2003	*B. psudocatenulatum* M115	*E.coli*	*B. breve* UCC2003	*B. psudocatenulatum* M115
1500V, 25μF, 200Ω	1.8×10^7	0	2.5×10^3	1.0×10^7	0	1.5×10^3
1800V, 25μF, 200Ω	ND*	0	4.8×10^4	ND*	0	1.2×10^4
2000V, 25 μF, 200Ω	ND	0	5.2×10^4	ND	0	ND
2500V, 25 μF, 200Ω	ND	0	ND	ND	0	ND

*ND: not done or not applicable.

6.6 Plasmid stability assay

The stability of two constructs including the pAM-P919-VP1-TTand pAM1-BetA-VP1-TT was assayed in *B. pseudocatenulatum* M115. In the absence of selective pressure, two constructs pAM-P919-VP1-TT and pAM1-BetA-VP1-TT vectors were maintained at percentages of 80 and 57 respectively, after 160 generations or 8 days. For pAM1 vector, the vector was maintained at percentages of 83 after 160 generations

(Figure 18). This result suggested that the constructs have satisfied segregational stability in *B. pseudocatenulatum* M115.

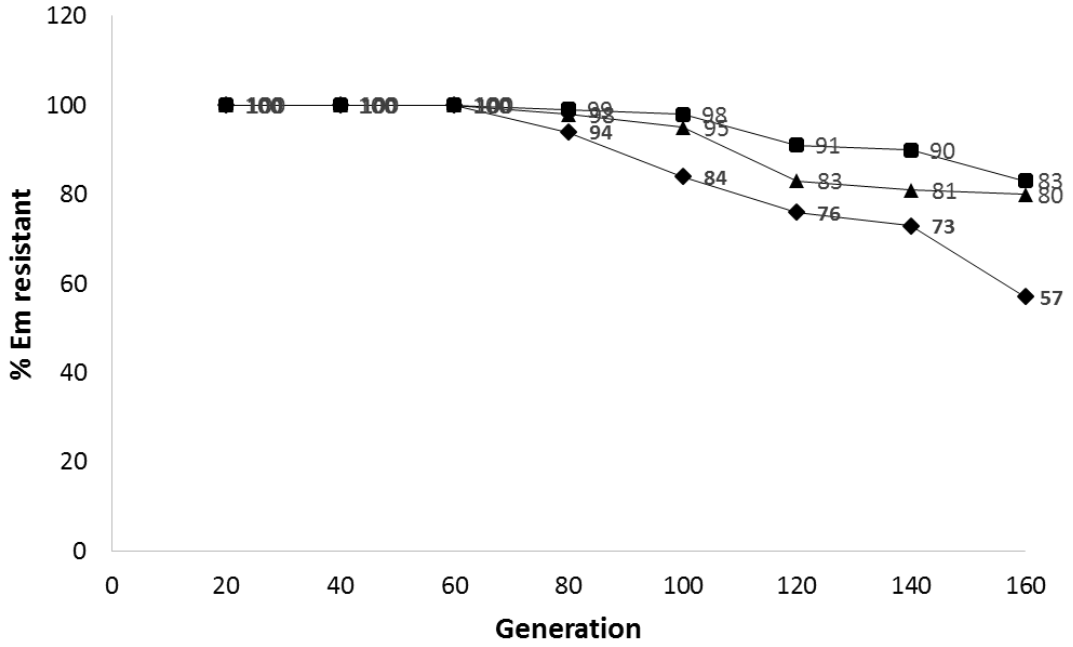


Figure 18 Segregational stability of the shuttle vectors pAM1 (■), pAM-P919-VP1-TT (▲) and pAM1-BetA-VP1-TT (◆) in *B. pseudocatenulatum* M115. *B. pseudocatenulatum* M115 harboring these plasmids was culture in the absence of selective pressure, plated on the same condition, and assayed for plasmid maintenance by replica-plating onto erythromycin-containing MRSC medium at approximately 20, 40, 60, 80, 100, 120, 140 and 160 generations intervals. Em^r, erythromycin.

6.7 Determination of expressed VP1 protein in bacteria under the control of pAM1-P919-VP1-TT

6.7.1 Determination of VP1 expression in recombinant *E.coli* XL1-blue containing pQE31-VP1

The bacterial lysates derived from the recombinant *E.coli* XL1-blue were determined for VP1 expression by western blot analysis. As shown in figure 19, the specific protein band of VP1 (34 kDa) was clearly detected in recombinant *E. coli* XL1-blue containing

pQE31-VP1 but not found in that containing plasmid pQE31. As shown in figure 20, VP1 protein band was found in both recombinant *E. coli* XL1-blue containing pQE31-VP1 and that containing pAM1-P919-VP1-TT but not in *E. coli* XL1-blue containing the empty plasmid pAM1. This result indicated that the P919 promoter located in pAM1-P919-VP1-TT could control the expression of VP1 in *E. coli*.

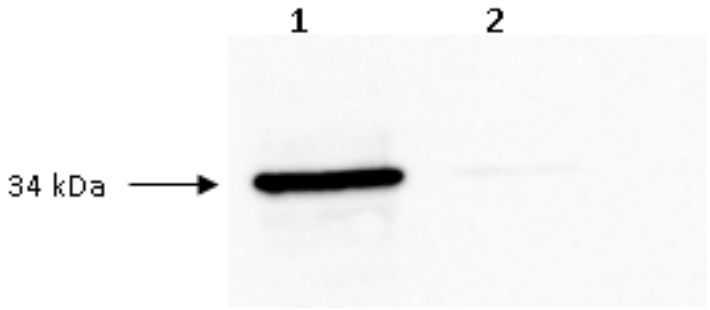


Figure 19 Immunodetection of the expressed VP1 protein in transformant *E. coli* XL1-blue by monoclonal antibody against VP1. Lane 1: *E. coli* XL1-Blue containing pQE31-VP1. lane 2: *E. coli* XL1-blue containing pQE31 use as negative control. The arrow indicates the VP1 band with the molecular weight of 34 kDa.

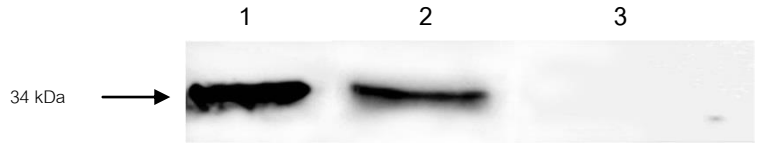


Figure 20 Immunodetection of the expressed VP1 protein in transformant *E. coli* XL1-blue by monoclonal antibody against VP1. Lane 1: *E. coli* XL1-Blue containing pQE31-VP1. lane 2: *E. coli* XL1-blue containing pAM1-P919-VP1-TT, lane 3: *E. coli* XL1-blue containing pAM1 used as negative control. The arrow indicates the VP1 band with the molecular weight of 34 kDa.

6.7.2 Determination of VP1 expression in recombinant *B. pseudocatenulatum* M115 containing pAM1-P919-VP1-TT

The recombinant expression plasmid pAM1-P919-VP1-TT was electrotransformed into *B. pseudocatenulatum* M115 for expressing VP1 protein. The bacterial lysates derived from *B. pseudocatenulatum* M115 was determined for VP1 expression by western blot analysis using mouse monoclonal antibody against VP1. As shown in figure 21, the VP1 protein with the molecular weight of 34 kDa can be detected in *E. coli* XL1-blue harboring recombinant expression plasmid pQE31-VP1 (pQE31-His is fusion vector for N-terminal 6xHis tag, Ap^r, for expressed as a 6xHis-tagged proteins) and *B. pseudocatenulatum* M115 harboring recombinant expression plasmid pAM1-P919-VP1-TT, but not in *B. pseudocatenulatum* M115 harboring plasmid pAM1 and in *B. pseudocatenulatum* M115. These results indicated that the recombinant *B. pseudocatenulatum* M115 can successfully express the VP1 protein under the control of P919 constitutive promoter.

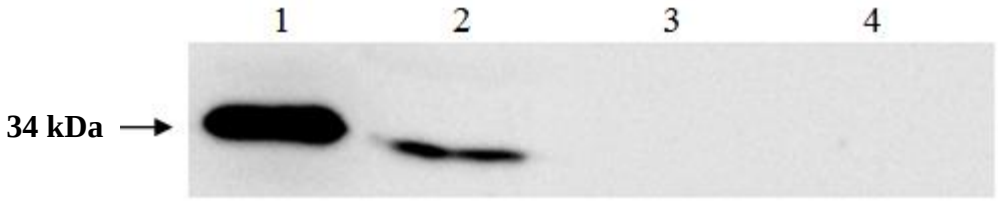


Figure 21 Immunodetection of the expressed VP1 protein in *B. pseudocatenulatum* M115 by monoclonal antibody against VP1. Lane 1: *E. coli* XL1-Blue: pQE31-VP1. Lane 2: *B. pseudocatenulatum* M115 harboring recombinant expression plasmid pAM1-P919-VP1-TT. Lane 3: *B. pseudocatenulatum* M115: pAM1. Lane 4: *B. pseudocatenulatum* M115. The arrow indicates the VP1 band with the molecular weight of 34 kDa.

6.8 Determination of expressed VP1 protein in *B. pseudocatenulatum* M115 under the control of pAM1-BetA-VP1-TT

The recombinant expression plasmid pAM1-BetA-VP1-TT was electrotransformed into *B. pseudocatenulatum* M115 for expressing VP1 protein. The bacterial lysates derived from *B. pseudocatenulatum* M115 was determined for VP1 expression by western blot analysis using mouse monoclonal antibody against VP1. As shown in figure 22, the VP1 protein with the molecular weight of 34 kDa could not be detected in *B. pseudocatenulatum* M115 harboring recombinant expression plasmid pAM1-BetA-VP1-TT. This result indicated that our study failed to induce the VP1 expression when using BetA promoter with different concentration of cholic acid, although several attempts were performed. Thus, for further construction of secretion system in *B. pseudocatenulatum* M115, the strong constitutive promoter P919 is used.

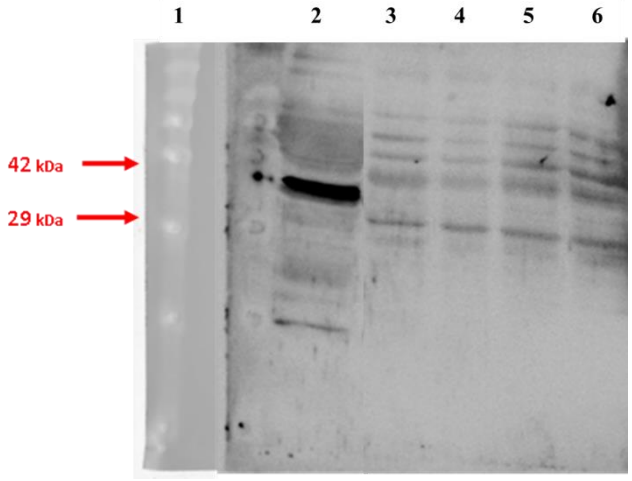


Figure 22 Immunodetection of the expressed VP1 protein in *B. pseudocatenulatum* M115 by monoclonal antibody against VP1. Lane 1: Protein marker. Lane 2: *E. coli* XL1-Blue: pQE31-VP1 (positive control). Lane 3: *B. pseudocatenulatum* M115 harboring recombinant expression plasmid pAM1-BetA-VP1-TT without the induction. Lane 4, 5 and 6: *B. pseudocatenulatum* M115 harboring recombinant expression plasmid pAM1-BetA-VP1-TT were induced with 0.1%, 0.08% and 0.5%, respectively, of cholic acid. Lane 4: *B. pseudocatenulatum* M115. The arrow indicates the standard protein band with the molecular weight of 29 and 42 kDa.

6.9 Construction of recombinant *B. pseudocatenulatum* M115 expressing VP1 in extracellular location

To construct the recombinant *B. pseudocatenulatum* M115 expressing VP1 in extracellular location The extracellular exo-xylanase (XynF) signal peptide (Xs) of *B. longum* (107) were amplified by PCR (Table6). The amplified product was inserted into the expression vector of both pAM1-BetA-VP1-TT and pAM1-P919-VP1-TT. The result fund that only the secretion-expression vector under the control of BetA inducible promoter named as pAM1-XynF-BetA-VP1-TT was obtained (Figure 24). The recombinant pAM1-XynF-BetA-VP1-TT was introduced into *E.coli* XL1-blue. The positive transformants were identified by *Eco*RI digestion. *Eco*RI -digested pAM1-XynF-BetA-VP1-TT generates two DNA fragments with the length of 6211 and 1800 bp. (Fig. 23, lane 1). Further verification of this constructs was done by sequencing (Three sequencing primers, I4-VP1c_ F 5' AAC CCG TCC GTG TTC GTG AA 3', I4-BetAc_F 5' ATG AAC CTG TAT CGC TAC GC 3' and I4-VP1s_R 5' AGT CGC CGA TGG AGG ACT 3', were designed and used for DNA sequencing). For the secretion-expression vector under the control of P919 constitutive promoter named as pAM1-XynF-P919-VP1-TT was not obtained. Due to the single mutation at signal peptide coding DNA sequence was always occurred after construction.

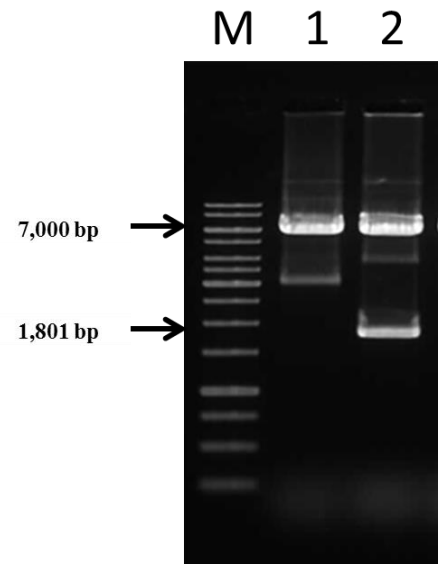


Figure 22 SYBR Gold staining gel of *Eco*RI-digested pAM1-XynF-BetA-VP1-TT. Lane M: 1 kb plus DNA ladder, lane 1: *Eco*RI-digested pAM1-XynF-BetA-VP1-TT:clone1 and lane 2: *Eco*RI-digested pAM1-XynF-BetA-VP1-TT:clone2.

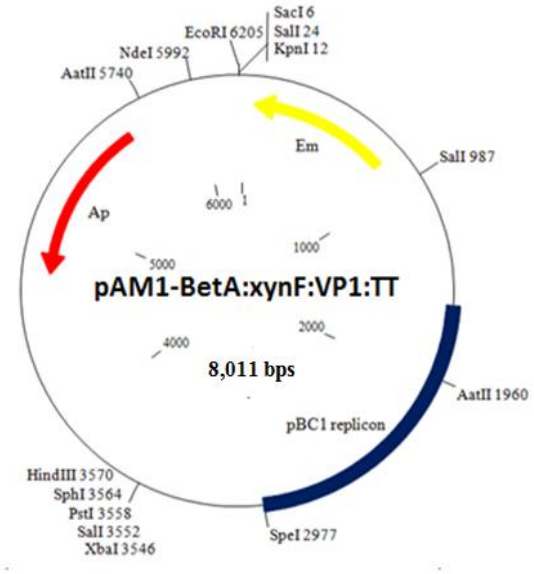


Figure 23 Physical and genetic map of the plasmid pAM1-BetA-xynF-VP1-TT (8,011 bp). The position of some key-restriction enzymes is also indicated**.**

6.10 Construction of recombinant *B. pseudocatenulatum* M115 expressing VP1 in cell wall anchorage location

To construct the recombinant *B. pseudocatenulatum* M115 expressing VP1 in cell wall location. The cell wall anchorage coding DNA sequence was amplified from genomic DNA extracted from of *Bifidobacterium longum* C35 (IPLA strain, a gift from Prof. Baltasar Mayo) by using the PCR primers set (809_CWA_F and 809_CWA_R, Table1). The amplified products were digested by XbaI and cloned into XbaI-digested pUCIDT-BetA-xynF-VP1-TT. The ligated DNA was transformed into *E. coli*. The positive transformants were identified by *Eco*RI and *Xba*I digestion. *Eco*RI - pUCIDT-BetA-xynF-VP1-TT generates two DNA fragments with the length of 2,940 and 2,650 bp. *Eco*RI - pUCIDT-BetA-xynF-VP1-TT generates two DNA fragments with the length of 4,651 and 849 bp. Further verification of this constructs was done by sequencing (Two sequencing primers, M13F-pUC(-40) and M13R-pUC(-26)) were used. This result indicated that plasmid vector harboring a fusion gene consisting of betA promoter, xynF signal peptide, VP1, CWA, and TT sequence, were obtained (Figure 25). For further work, this fusion gene could be cloned into pAM1 vector.

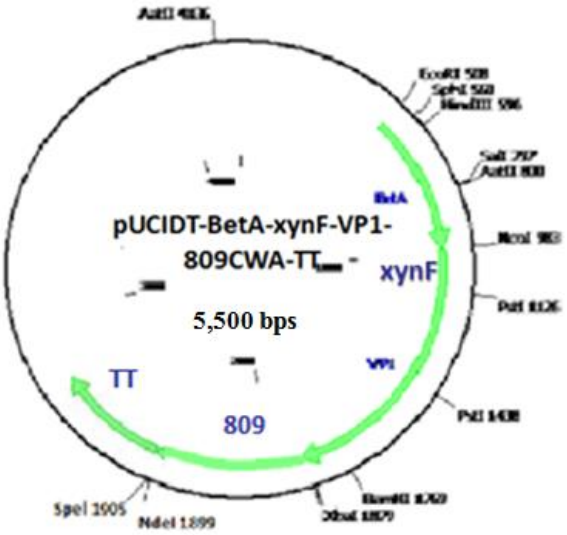


Figure 25 Physical and genetic map of the plasmid pUCIDT-BetA-xynF-VP1-809CWA-TT (5,500 bp). The position of some key-restriction enzymes is also indicated**.**

7. Discussion and Conclusion

The enterovirus 71 (EV71) has been reported as causative viral pathogens of hand-foot-and-mouth disease (HFMD) in infants and young children. The infection occasionally caused a severe neurological complication and leaded to dead. EV71 had emerged as a serious threat through the Asian countries, including Thailand [88-90]. Currently, various vaccines against EV71 are being investigated including inactivated whole viruses, live attenuated virus, viral-like particle, subviral particle, and DNA vaccine. However, no effective vaccine against EV71 has been reported. Thus, further vaccine development against EV71 infection is needed. Four structural of viral capsid proteins (VP) of EV71, i.e. VP1, VP2, VP3, and VP4 have been shown to be the good immunogens, but VP1 seems to be the most potential antigen for incorporating in vaccine formulation, as it has been demonstrated to elicit protective neutralizing antibodies and resulting in prevention of infection [91-95]. Compared with the parenteral route of vaccination, mucosal immunization offers several advantages over parenteral immunization such as elicit local and distal mucosal as well as systemic immune responses.

Bifidobacterium species, the predominant bacteria found in human gastrointestinal tract, are considered as probiotic due to their beneficial effects on human health [96-98]. Recombinant *Bifidobacterium* expressing reactive antigens derived from pathogens have been constructed and used for immunization in animal model to protect against many infectious diseases [11, 96, 97, 99, 100]. It has been reported that recombinant *B. longum* expressing EV71-VP1 could induced neutralizing antibodies and Th1 cytokine against VP1 in mice. In addition, it can elicit both mucosal and systemic immune responses. Such finding indicated that *B. longum* might be used as bacterial vehicle for delivery of VP1 protein for mucosal immunization in human [101, 102].

In this study, *B. pseudocatenulatum*M115 was selected to construct the recombinant *B. pseudocatenulatum*M115 for the expression of EV71-VP1 protein in

intracellular location. The localizations of the expressed antigen in bacterial cell, i.e. cytoplasm, secreted and cell wall anchored locations, can have influenced on the types of immune responses and on its own immunogenicity. The expression of various antigens on different location have been reported for their success in eliciting immune response, but no conclusive evidence to indicate which location of expressed antigen should be located have been reported [103].

In this study, the VP1 capsid protein of enterovirus 71 was used as a model antigen for expression in *B. pseudocatenulatum*M115. From our analysis, its codon usage is considerable different from that of Bifidobacterium species. As the codon usage is one important factor that affects the expression efficiency. Therefore, the native VP1 coding sequence has been changed to that preferrently used by the expression bacterial host i.e. *Bifidobacterium* species.

For the control of protein expression, the BetA inducible promoter derived from *B. longum* NCC2705 is the first promoter used in this study. This inducible promoter has been used for the expression of arabinofuranosidase when inducing with cholic acid but no expression without this inducer. [104, 105]. Unfortunately, our study failed to induce the VP1 expression when using BetA promoter with cholic acid induction. The failure of BetA system led us to change the promoter to P919 which is strong constitutive promoter. Previous study demonstrated that P919 promoter derived from *B. bifidum* BGN4 showed the highest efficiency for s control the expression of β-glucosidase in both *B. bifidum* BGN4 and *E. coli* DH5α [58]. Therefore this promoter has been selected to incorporate in our recombinant expression plasmid for VP1 expression in bifidobacterium species

Besides the efficient promoter, the transcription terminators (TT) is needed [106]. Transcription terminator was inserted at downstream of VP1 gene in recombinant plasmid pUCIDT-P919-VP1. To produce recombinant expression plasmid containing fragment of P919-VP1-TT, pAM1 shuttle vector was used in this study. Based on the result of immunodetection using monoclonal antibody against VP1, the protein band

with the molecular weight of 34 kDa was detected in the crude lysate protein. This result indicated that the recombinant *B. pseudocatenulatum* M115 harboring pAM1-P919-VP1-TT could express the VP1 protein in intracellular location. In conclusion, we have successfully constructed the recombinant *B. pseudocatenulatum* M115expressing EV71-VP1 protein as intracellular location. The VP1 nucleotide sequence was compared with that of the codon usage of *B. breve* UCC2003. However, this codon usage could also be used for VP1 expression in *B. pseudocatenulatum* M115. The strong constitutive promoter derived from bifidobacterium, P919 can be used for control protein expression as intracellular location. This recombinant *Bifidobacterium* can further be used for construction the recombinant *Bifidobacterium* expression of EV71-VP1 protein in different location i.e. extracellular and cell wall anchorage location. Such recombinant bacteria expressing VP1 in different locations are useful for immunization study against Enterovirus 71 infection in the future.

In conclusion, in this study, several expression vectors were successfully constructed, although only intracellular expression was successfully verified. For extracellular and cell wall expression, some plasmid vectors, e.g. secretion-expression vector has been already constructed, but the VP1 protein verification has not been successfully finished in this project. To finish this aim, further verification of VP1 is required. Finally, The recombinant *B. pseudocatenulatum* M115 expressing VP1 protein in intracellular might be valuable tools for the further evaluation of their immunogenicity in eliciting the immune response in animal model.

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APPENDICE

APPENDIX A

Chemicals, Enzymes and Antibiotics

1. Chemical

All chemicals used in the study were analytical grade. Name and sources of chemicals are listed below.

Chemicals	**Sources**
Absolute ethanol	Scharlau
Acetic acid glacial	BDH Laboratory supplies
Acrylamide	Amersham Pharmacia Biotech
Agarose	Life Technologies Inc.
Ammonium acetate	Famitalia Carlo Erba
Ammonium citrate	Famitalia Carlo Erba
Ammonium persulfate	Amresco Inc.
Chloroform	Amresco Inc.
Chromatein Prestained protein-Ladder	Vivantis
Cystein	Sigma
D-glucose	Scharlau
Diaminoethane tetra-acetic acid (EDTA)	Fisher Chemical
Dipotassium hydrogen phosphate	Fluka
Disodium hydrogen phosphate	Fluka
Ethidium bromide	Sigma Chemical Co.
Glycerol	Sigma Chemical Co.
Hydrochloric	Merck
IPTG	Fermentas
Iso-amyl alcohol	Univar
N, N methyl-bis-acrylamide	Amersham Pharmacia Biotech
PageRulerTMprestrained Protein-Ladder	Fermentas Life Sciences
Phenol	Ameresco Inc.
Polyethylene glycol 8000	USB
Potassium acetate	BDH Laboratory supplies
Potassium chloride	BDH Laboratory supplies
Sucrose	USB
Sodium chloride	Sodium dodecyl sulphate (SDS)

Chemicals	**Sources**
Famitalia Carlo Erba	Sigma Chemical Co
Sodium hydroxide	Famitalia Carlo Erba
TritonX-100	Fluka
TEMED (Tetramethylenediamine)	Merk
Tris (Hydroxymethyl amino methane)	Merk
Tween 20	Amresco

2. Enzymes

All enzymes used in this study were analytical grade. Names and sources of enzymes are listed below

Enzymes	**Sources**
*Eco*RI	Fermentas Life Sciences
*Nco*I	Fermentas Life Sciences
*Xba*I	Fermentas Life Sciences
*Nde*I	Fermentas Life Sciences
Lysozymes	Sigma Chemical Co.
Mutanolysis	Sigma Chemical Co.
Taq DNA polymerase	Biotool

3. Antibiotics

Antibiotics	**Sources**
Ampicillin	Dumex
Erythromycin	Fluka

APPENDIX B

Culture media and Buffers

1. Media for *Bifidobacterium*

1.1 10% Cysteine

Cysteine	10 g

Add DW to final volume of 100 ml and filtered sterile.

1.2 MRSC medium

MRS	55 g

Add DW to final volume of 1000 ml. Autoclave at 121˚C and 15 min. After autoclave, add 0.25% Cysteine. .

1.3 MRSC agar

MRS	55 g
Agar	15 g

Add DW to final volume of 1000 ml. After autoclave, add 0.25% Cysteine.

2. Media for *Escherichia coli*

2.1 LB medium

Bacto tryptone	16 g
Bacto yeast extract	10 g
NaCl	5 g

Add DW to final volume of 1000 ml

2.2 LB agar

Bacto tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g
Bacto agar	15 g

Add DW to final volume of 1000 ml

3. Reagents for agarose gel electrophoresis

3.1. Agarose gel preparation

1% agarose	1 g in 100 ml of 1X TBE buffer

3.2 50X Tris-acetate/EDTA buffer (TAE buffer)

Tris base	242 g
Glacial acetic acid	57.4 g
0.5 M EDTA pH 8.0	200 g
Add DW to a final volume of 1000 ml	

4. Reagents for plasmid preparation

4.1 *E. coli* plasmid

4.1.1 Glucose solution

1 M Tris-HCl pH 8.0	2.5 ml
1 M glucose	5.0 ml
500 mM EDTA	2.0 ml
Add DW to a final volume of 100 ml	

4.1.2 Lysozyme solution

Glucose solution	800 µl
Lysozyme	10 mg
10 mg/ml RNAse A	20 µl
Freshly preparation lysozyme solution	

4.1.3 NaOH/SDS solution

10 M NaOH	0.1 ml
10 % SDS	0.5 ml
Sterilized distill water	0.4 ml
Freshly preparation NaOH/SDS solution	

4.1.4 K solution

5 M Potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Add DW to final volume of 100 ml	

4.1.5 phenol:chloroform:isoamyl alcohol (25:24:1)

4.1.6 70 % ethanol

Absolute ethanol	70 ml
Add DW to final volume of 100 ml	

4.2 Lactic acid bacteria plasmid

4.2.1 STE buffer

1 M Tris-HCl, pH 8.0	2.5 ml
0.5 M EDTA	5 ml
Sucrose	10.3 g

Add DW to final volume of 100 ml

4.2.2 Alkaline lysis solution

10 % SDS	3 ml
1 N NaOH	2 ml

Add DW to final volume of 10 ml

4.2.3 3M sodium acetate (pH 4.8)

4.2.4 Isopropanol

5. Buffer for competent cells preparation and electroporation

5.1 *E. coli*

5.1.1 SOB medium

Bacto tryptone	20 g
Bacto yeast extract	5 g
NaCl	0.5 g
1 M KCl	2.5 ml

Add DW to final volume of 1000 ml and 1 ml of MgCl2 to each 100 ml aliquot.

5.1.2 SOC medium

Add 1 ml of 1 M filtered sterile glucose into 100 ml of SOB medium

5.2 *Bifidobacterium* (Sucrose-Ammonium citrate)

5.2.1 0.5 M Sucrose

0.5M Sucrose	17.12 g

Add DW to final volume of 100 ml and filtered sterile.

5.2.2 1mM di-ammonium citrate (pH 5.8)

1mM di-ammonium citrate 0.23 g

Add DW to final volume of 10 ml and filtered sterile.

6. Reagent for protein extraction

5.1 Sonication buffer

Stock reagent	Final concentration	For 10 ml
500 mM NaH2PO4	50 mM	1 ml
5 M NaCl	300 mM	600 µl
1 M Imidazole	10 mM	50 µl
100 mg/ml Lysozyme	1 mg/ml	100 µl
100X Proteinase inhibitor	1X	100 µl
1 M DDT	10 mM	50 µl
Sterile distilled water		8.15 ml

Adjust the pH to 8.0 using NaOH

7. Reagent for SDS-PAGE

7.1 30% acrylamide solution (per 100 ml)

Acrylamide 29 g

Bis-acrylamide 1 g

Add DW to final volume of 100 ml and store at 4˚C

7.2 Gel buffer pH 8.8

Tris 36.3 g

Adjust to pH 8.8 with 1N HCl

Add DW to final volume of 100 ml

7.3 Gel buffer pH 6.8

Tris 36.3 g

Adjust to pH 6.8 with 1N HCl

Add DW to final volume of 100 ml

7.4 10X Electrophoresis buffer

Tris	36.28 g
Glycine	144.14 g
SDS	10 g

Add DW to final volume of 1000 ml

7.5 Staining solution

Coomassie brilliant blue R250	1 g
Absolute methanol	250 ml
Glacial acetic acid	10 ml

Add DW to final volume of 500 ml

7.6 Destaining solution

Absolute methanol	100 ml
Glacial acetic acid	140 ml
Add DW to final volume of	200 ml

7.7 10% Ammomium persulfate

Ammomium persulfate 0.5 g

Add DW to a final volume 5 ml. This solution should be freshly prepared before used.

8. Sample buffer

0.5 M Tris-Hcl, pH 6.8	1.25 ml
Deionized water	5.55 ml
Glycerol	3 ml
0.5% (w/v) Bromophenal blue	0.2 ml
Total volume	10 ml

9. Acrylamide Gel preparation

Reagents	**5 % gel**	**15 % gel**
Stocking acrylamide 30%	1.66 ml	4.98 ml
Gel buffer pH 6.8	1.25 ml	-
Gel buffer pH 8.8	-	1.25 ml
10% SDS	100 µl	100 µl
DW	6.67 ml	3.35 µl
TEMED	10 µl	10 µl
10% APS	300 µl	300 µl
Total	10 ml	10 ml

10. Reagent for Western blot

10.1 Blocking buffer

BSA	3 g

Add TBS tween buffer to a final volume 100 ml

10.3 Stock Semi-dry blotting buffer

Tris-base	15.15 g
Glycine	72 g

Add DW to final volume of 500 ml and store at 4˚C

10.4 Semi-dry blotting buffer

Methanol	200 ml
DW	700 ml

Added stock Semi-dry blotting buffer 100 ml and store at 4˚C

10.5. Tris-buffered saline (TBS) buffer

1M Tris-HCl pH 7.5	10 ml
5M NaCl	50 ml

Add DW to final volume of 500 ml

10.6 TBS tween/triton buffer

1M Tris-HCl pH 7.5	10 ml
5M NaCl	50 ml
Tween 20	0.25 ml

Add DW to final volume of 500 ml

APPENDIX C

PCR Amplification Condition

Table 6 PCR conditions used for amplification of VP1, P919, TT, XynF, and CWA DNA

Target gene	Primers[a]		DNA template	Amplification condition	Times (sec or min)	Cycles no.	Amplicon Size (bp)
	Forward	Reverse					
VP1	VP1-F	VP1-R	pUCIDT-BetA-VP1	Pre-denaturation (95°C)	5 min	1	891
				Denaturation (95°C)	30 sec	30	
				Annealing (60°C)	40 sec		
				Extention (72°C)	1 min		
				Final extention (72°C)	7 min	1	
P919	P919-F	P919-R	ProSP_New (gBlocks 429 base pairs)	Pre-denaturation (95°C)	5 min	1	206
				Denaturation (95°C)	30 sec	30	
				Annealing (56°C)	30 sec		
				Extention (72°C)	20 sec		
				Final extention (72°C)	7 min	1	
TT	TT-F	TT-R	pLC13.9-Ldh-M2e:HBc-TT	Pre-denaturation (95°C)	5 min	1	332
				Denaturation (95°C)	30 sec	30	
				Annealing (55°C)	30 sec		
				Extention (72°C)	40 sec		
				Final extention (72°C)	7 min	1	
XynF	XynF_F	XynF_R	ProSP_New (gBlocks 429 base	Pre-denaturation (95°C)	5 min	1	114
				Denaturation (95°C)	30 sec	30	

			pairs)	Annealing (55°C)	30 sec		
				Extention (72°C)	40 sec		
				Final extention (72°C)	7 min	1	
CWA	809_CWA _F	809_CWA _R	Genomic DNA of B. longum C35	Pre-denaturation (95°C)	5 min	1	850
				Denaturation (95°C)	30 sec	30	
				Annealing (55°C)	30 sec		
				Extention (72°C)	40 sec		
				Final extention (72°C)	7 min	1	

[a] Primers sequence are shown in Table 2.

Research publication

1. Tapanee Thinbanmai, Viraphong Lulitanond, Baltasar Mayo, Aroonlug Lulitanond, **Marutpong Panya.** Cloning and expression of enterovirus 71 capsid protein 1 in Bifidobacterium pseudocatenulatum. (**Submitted Manuscript, Attached file_1**).
2. Tapanee Thinbanmai, **Marutpong Panya**, Aroonlug Lulitanond, Hlaing Hlaing Thaw, Baltasar Mayo, Virapong Lulitanond. Construction of recombinant plasmid for intracellular expression of enterovirus 71 viral capsid protein (vp1) in *Bifidobacterium breve* UCC2003. The 8th Asian Conference on Lactic Acid Bacteria 2015, The emerald hotel, Bangkok, Thailand. (**Poster presentation, Attached file_2**).
3. Tapanee Thinbanmai, **Marutpong Panya,** Aroonlug Lulitanond, Baltasar Mayo, Viraphong Lulitanond. Construction of recombinant plasmids for expression of enterovirus 71 viral capsid protein 1 in *Escherichai coli* XL1-blue. International Conference on Learning Innovation in Science and Technology (ICLIST2016) January 27-29, 2016, Pattaya, Thailand. **(Proceeding**, **Attached file_3**).

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