Isolation of Gene(s) Encoding Polar Flagellar Core Antigen of *Vibrio parahaemolyticus*

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Abstract

A genomic library of *V. parahaemolyticus* was constructed in order to isolate the gene encoding polar flagellar core antigen. DNA fragments were cloned into Lambda ZAPII vector at the *Eco*RI cloning site and the recombinant DNA was transformed into *Escherichia coli* XL1-Blue strain. Recombinant plaques were screened with rabbit polyclonal antiserum produced against polar flagella and with monoclonal antibody produced against polar flagellar core protein. The pBluescript phagemid which contained the DNA fragment encoding the flagellar gene was rescued and analyzed. Additional investigation was carried out by mini-Mu transposon mutagenesis to map the coding region. The flagellar core coding region was mapped within 1.1 kb which encompasses the *Nco*I site present in the 6.5 kb insert.

1. Introduction

Unlike *Vibrio cholerae*, little or no information is available on the genetics of *V. parahaemolyticus*. This organism was reported to have mol% G+C for chromosomal DNA = 46-47 (*Tm*, Bd). In 1984, Belas et al. [1], studied the mechanism of chitin adhesion by the lateral flagella and developed mutants for lateral flagella (*laf*) expression of *V. parahaemolyticus* by using a mini-Mu transposon mutagenesis. This particular gene system was chosen partly because mutant phenotypes were convenient to recognize. Transposition resulted in transcriptional fusion of *Vibrio* genes with the transposon *lacZ* gene. Therefore, expression of the *lacZ* gene was proportional to the level of transcription for the target gene. Regulation of lateral flagella gene expression was studied *in vivo* by measuring β-galactosidase activity and the conditions which activated transcription of these genes were identified. Subsequently, Belas et al. [2] studied the regulation of lateral flagella gene (*laf*) expression by constructing fusions between the *laf* gene and the indicator luminescence gene (*lux*). They found that the expression of lateral flagella synthesis is surface-dependent and controlled by regulation of transcription. They also concluded that changes in viscosity of the medium are sufficient to induce all the phenotypic changes associated with swarmer cell differentiation.

In this investigation, genomic libraries of *V. parahaemolyticus* were constructed into Lambda ZAPII vector. The plaques were screened with flagellar core-specific polyclonal and monoclonal antibodies. Mini-Mu (15.8kb) transposon was used to mutagenize the flagellar gene generating null mutation. The insertion of several kilobases of the transposon permits precise physical mapping for the location of the mutation. The ultimate goal is to compare the homology of DNA sequences encoding polar

flagellar core proteins between *V. parahaemolyticus* and *V. vulnificus*.. We are interested in the flagellar core proteins because they express species-specific antigenic determinants. Their homology may provide insight into preparation of probes which would be useful in the construction of a detection system.

2. Materials and Methods

2.1 Construction of *V. parahaemolyticus* genomic DNA library

2.1.1 Bacteria and phage : Bacterial strains, plasmids, and bacteriophages are listed in Table 1. *Vibrio parahaemolyticus* ATCC 10136 DNA was used as a source of genomic DNA.

2.1.2 Chemicals, media and culture condition : *Escherichia coli* strains were maintained on Luria-Bertani (LB) medium containing appropriate antibiotics listed on Table 2. The antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo. Recombinant phages were plated onto NZY [5 gm NaCl, 2 gm $MgSO_4.7H_2O$, 5 gm yeast extract, 10 gm NZ Amine (casein hydrolysate), 15 gm Bacto-agar per liter, pH 7.5] agar plates.

2.1.3 DNA purification : DNA of *V. parahaemolyticus* ATCC 10136 was purified by a method modified from Murmur [3] and Ford [4]. Two 100 ml-flasks of alkaline peptone broth (10 gm Bacto-peptone, 20 gm sodium chloride, 2 gm yeast extract, 4 gm $MgCl_2.H_2O$, 4 gm KCl per liter, pH 7.5) were inoculated from the maintenance culture and incubated at 37°C for 5-6 hrs at 200-250 rpm on a rotary shaker. Each culture was used to inoculate one liter of the same medium and incubated at 37°C at 200-250 rpm overnight. A Gram stain from each culture was prepared and examined.

Cells were sedimented by centrifugation at 13,000 x g for 20 min at 4°C and were stored at -20°C until needed. The cell pellets were thawed in a 37°C water bath and suspended in 50 ml saline-EDTA (0.15 M NaCl + 0.1 M EDTA, pH 8.0). The cells were lysed by addition of 25% sodium dodecyl sulfate (SDS) and sodium perchlorate (NaClO4) to provide high salt concentration for the subsequent step. Deproteinization was done twice by extraction with chloroform:isoamyl alcohol at a ratio of 24:1 (v/v). The DNA extract was precipitated with two volumes of cold ethanol. The precipitate was spooled and redissolved in small volume of Tris-EDTA (10 mM Tris + 1 mM EDTA, pH 8.0) buffer. Finally, the absorbances at 260 nm and 280 nm were determined by spectrophotometry. A value of O.D. 260:280 = 1.8 suggests that protein and ribonucleic acid have been removed and the concentration of the purified DNA content was calculated by reading O.D. 260 (1 OD_{260} = 50 μg/ml).

Rapid, small-scale plasmid isolation was performed by the method described by Birnbom and Doly [5].

2.1.4 Cloning of *Vibrio parahaemolyticus* gene encoding polar flagellar protein(s) : Genomic DNA from *Vibrio parahaemolyticus* was partially digested with *Eco*RI and ligated into the *Eco*RI sites of Lambda ZAPII vector. The recombinant DNA was packaged into Lambda ZAPII by using the Gigapack II Packaging Extract (*hsd*, *mcrA*-, *mcrB*-, and *mrr*-) (Stratagene, La Jolla, CA). The efficiency of packaging and recombination were evaluated by estimating the ratio of blue and white plaques upon plating. The DNA library was amplified prior to screening.

2.1.5 Production of anti-H polyclonal antibodies : Twelve to 16 week-old New Zealand white rabbits were immunized intravenously with a suspension of *V. parahaemolyticus* ATCC 10136 cells (10^{10} cells/ml). One month following the first immunization, the rabbit was exsanguinated and the serum titer was determined by H-flocculation [6]. Antiserum obtained by this immunization schedule produced low titer (1:160 to 1:640) by H-flocculation and produced weak reactions when used to screen the DNA library. Therefore a second protocol was attempted.

Table 1. Bacterial strains and bacteriophages used in this investigation.

Bacterial strains, and bacteriophages	Description	References
V.parahaemolyticus ATCC 10136	wild type	American type culture collection
E. coli		
XL1-Blue (Stratagene, La Jolla, CA)	*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17(*rk*-,*mk*+), *sup*E44, *rel*A1, λ-, *lac*-, [F', *pro*AB, *lac*I^{q}Z_M15, Tn10(tet)]	Bullock et al., 1987 [7]
POI1681	*ara*D, *ara*B::Mu *cts*, *lac*, *rec*A, *rsp*L	Castiho et al., 1984 [8]; Belas et al., 1984 [1]
MH3497	Rec^{+}, Mu *cts*	M. Howe, University of Wisconsin, Belas et al., 1984 [1]
Bacteriophage		
λ ZAP II (Stratagene, La Jolla, CA)	λ ZAP, *S*+ gene from λ gt10	Short et al., 1988 [9]

Table 2. List of antibiotics used in bacterial strains, vectors, and transposon.

Bacterial strains, vectors, and transposon	Antibiotics
XL1-Blue	Tetracyclin (12.5 μg/ml)
POI1681	Kanamycin (25 μg/ml)
MH3497	none
Lambda ZAPII	Ampicillin (50 μg/ml)
pBluescript SK-	Ampicillin (50 μg/ml)
Mini-Mu (Mu dI1681)	Kanamycin (25 μg/ml)

Five hundred micrograms of purified *V. parahaemolyticus* flagella were mixed with an equal volume of Freund's complete adjuvant and one ml of the emulsion was injected intradermally in 14 to 16 sites on a shaved rabbit back. One month following immunization, the rabbit was test bled and the H titer was 1:640. At this time, 0.5 ml purified flagella (300 μg) and 0.5 ml Freund's incomplete adjuvant were mixed and injected intramuscularly at 2 sites. Seven weeks following the first immunization, the H titer was 1:5120 and the rabbit was exsanguinated.

2.1.6 Production of anti-H monoclonal antibodies (MAb-flc) : MAb-flc (clone 4G7), obtained from a previous experiment was purified by Protein G (Pharmacia LKB Biotechnology, Piscataway, NJ) column chromatography. Both rabbit antisera and mouse ascites fluid were absorbed with a sodium azide-killed *E. coli* cell suspension and/or treated with commercially

available *E. coli* extract (Promega Corporation, Madison, WI) to remove background IgG components that bind to *E. coli* proteins [10].

2.1.7 Immunoscreening of the recombinant phages : The *V. parahaemolyticus* DNA library was screened for flagellar core protein by using ProtoBlot Immunoscreening System (Promega Corporation, Madison, WI). The DNA library, in *E. coli* XL1-Blue, was plated onto Petri dishes for screening (Undigested Lambda ZAPII Cloning Kit; instruction manual, Stratagene Cloning Systems, La Jolla, CA). The culture to be plated was started from a single colony of XL-1 Blue by propagation overnight at 30°C with vigorous shaking in 25 ml LB medium supplemented with 0.2% maltose and 10 mM $MgSO_4$ (No tetracycline was added in the presence of Mg). Cells were sedimented at 1000 x g for 10 min and resuspended in 0.5 volume of 10 mM $MgSO_4$. Before plating, the cells were diluted to OD_{600} = 0.5 with 10 mM $MgSO_4$.

One hundred-millimeter plates were used to screen plaques for *V. parahaemolyticus* flagellar protein expression. Two-tenth ml of the XL-1 blue culture, previously adjusted to OD_{600} = 0.5, was mixed with 0.1 ml of the packaged lambda clones or amplified suspension which contained approximately 17,000 recombinant bacteriophage. This mixture was incubated for 15 min at 37°C without shaking. Each aliquot of the mixture was mixed with 3 ml melted (48°C) NZY top agar and poured evenly onto previously prepared NZY plates. The plates were incubated at 42°C for 3-3.5 h. Plates were removed from the incubator and each plate was overlaid with a dry nitrocellulose filter disk previously saturated in 10 mM IPTG. The plates were incubated at 37°C for 3.5 h. Next, the position of the filter on the plates was marked with a needle and the filter was removed.

Immunoscreening was done following the Promega Protocol [10]. Anti-rabbit conjugate (Promega Corporation, Madison, WI) was used at 1:7500 and anti-mouse conjugate (Organon Teknika corporation, Durham, NC) was used at 1:5000 throughout. The plaques which corresponded to positive signals on the filter pad were removed as agar plugs with sterile Pasteur pipettes. They were placed in microcentrifuge tubes in 1 ml of SM buffer, vortexed occasionally and stored at 4°C .

2.1.8 *In vivo* excision of recombinant pBluescript phagemid : This was done as described by the manufacturer (Stratagene, La Jolla, CA).

2.2 Transposon mutagenesis

The *E. coli* XL1-Blue strain containing the pBluescript phagemid was used as a source of DNA. The strain that provided the mini-Mu bacteriophage was *E. coli* strain POI1681 (*ara*D, *ara*B::Mu *cts*, *lac*, *rec*A, *rsp*L) [1, 8, 11]. The recipient which is capable of homologous recombination was *E. coli* strain MH3497 (*lac gal rps*L Mu *cts*) [1, 11].

2.2.1 Transformation of recombinant pBluescript phagemid into *E. coli* POI1681 carrying mini-Mu transposon : The pBluescript phagemid containing the insert was purified by the miniplasmid preparation method [5]. Conventional transformation was attempted however no transformant was obtained. Therefore electroporation of plasmids into the recipient cells was introduced. The protocol used was based on that described in "Bacterial Electro-transformation and Pulse Controller Instruction Manual" (Bio-Rad Laboratories, Richmond, CA).

DNA used for electroporation was prepared by minipreparation methods [5] and further purified by GENECLEAN II kit (Bio 101 Inc., La Jolla, CA). DNA concentration was determined by the absorbance at 260 nm. Prior to transformation, the cells were thawed at room temperature and placed on ice. In a cold 1.5-ml microtube, 40 µl of the cell suspension was mixed with 1-2 µl of DNA (in H_2O) and set on ice for 0.5 to 1.0 min. The mixture was

transferred to a cold 0.2 cm electroporation cuvette and pulsed once at the following settings; 25 μF, 2.5 kV, 200 Ω. Immediately, thereafter 1 ml of LB medium was added to the cuvette, and the cells were resuspended and then transferred into a clean tube. The cell suspension was incubated at 28 to 30 °C for 1 h to allow expression of the antibiotic resistance gene and the 100 μl was plated onto LB-Amp-Kan plates. The plates were incubated at room temperature until the colonies were observed. Several colonies were picked to prepare plasmid by minipreparation method and examined for the plasmid of interest.

2.2.2 Preparation of Mu-phage-transducing lysate by heat induction : The method was modified from that described by Casadaban and Cohen [12]. The transformants which were lysogens of Mu *cts* were propagated at 28° to 30°C. The cultures, in preparation for thermoinduction, were grown in LB medium to early exponential phase (OD_{600} = 0.2-0.3) at 28-32°C, then shifted to 42°C for 20 min without shaking, and then incubated at 37°C until lysis. Chloroform (1%, vol/vol) was added and the lysate was stirred. After 5 min, the lysate was stirred one more time and sedimented to pellet cell debris at 10,000 rpm for 10 min in Beckman SS34 rotor. To stabilize the Mu lysate, 1 M $MgSO_4$ was added to give a final concentration of 10 mM. Since Mu lysates can lose infectivity rapidly, they were used within a few days.

2.2.3 Transduction of Mu phage-transducing lysate into Rec+ *E. coli* strain : The Mu phage-transducing lysates were used to transduce Rec+ *E. coli* MH3497 as described previously [8, 12, 1, 13]. The location and orientation of the mini-Mu inserts in the mutated plasmids were determined by restriction mapping by utilization of the asymmetry of the *Eco*RI [8] and *Nco*I sites (observed in this study) of the transposon.

2.2.4 Induction of expression of the fusion protein by IPTG : In *E. coli* XL1-Blue, the cloning sites are in the N-terminal region of *lacZ* gene. Thus, the DNA sequence cloned into the multiple cloning sites of the λZAPII vector may be expressed as a fusion protein under the control of *lacZ* promoter which can be induced by IPTG. The protocol used for IPTG induction was modified from that described by Stark [14]. Cells were grown overnight at 37°C (for XL1-Blue) or 28-30°C (for MH3497) in LB medium containing the appropriate antibiotics and 0.5% glucose. Cells were then subcultured into fresh medium supplemented with antibiotics and glucose and then grown to OD_{600} of 0.5. Following centrifugation, the cells were resuspended to 0.5 OD_{600} in pre-warmed LB medium which contained antibiotics only. IPTG was added to a final concentration of 0.1 mM and the culture was shaken at 28° to 30°C for MH3497 strain or 37°C for XL1-Blue strain. Samples were taken from each culture at 0 (before addition of IPTG), 1, 2, 3, 4, and 5 h for strains which carried recombinant plasmid and at 0, and 4 to 5 h for the transductants, following addition of IPTG.

2.2.5 Screening of transductants by Western blotting : A 12% SDS-PAGE slap gel was prepared by the method of Laemmli [15]. The protein concentration for each sample was determined by BCA protein assay (Pierce, Rockford,IL) and approximately 8 μg protein of each sample was loaded onto the gel. Following the electrophoresis run, the gel was blotted onto nitrocellulose membrane (NC) overnight at 30 volts in the Trans-Blot system (Biorad Laboratories, Richmond, CA). Prior to removing the gel, the voltage was increased to 60 volts for 2 h. The NC was rinsed in TBST buffer and exposed to a 1:50 dilution of MH3497-absorbed MAb-flc.

2.2.6 Preparation of MH3497-absorbed antibody : *E. coli* MH3497 cells were grown in 200 ml of LB medium at 37°C overnight. The cells were sedimented and resuspended in 1% sodium azide (NaN_3) and incubated for 2-3 h. The cell suspension was aliquoted into three equal volumes which were centrifuged at 8000 rpm for 10 min in a

Beckman SS34 rotor. A 1:50 dilution of tissue culture supernatant fluid which contained MAb-flc was used to resuspend one volume of the NaN_3-killed cell pellet which was incubated 2-3 h before centrifugation. This absorption step was repeated two more times with the remaining aliquots. The absorbed antibody was stored at 4°C until needed.

2.2.7 Site mutagenesis at *Nco*I site located in the *Eco*RI insert fragment : The recombinant plasmid was purified and digested with *Nco*I. The *Nco*I hanging ends were filled in by Klenow fragment to create blunt ends and then religated. After ethanol precipitation, the plasmid was digested with *Nco*I before it was transformed into *E. coli* XL1-Blue strain. Finally, the recipient cells which contained this plasmid were IPTG-induced to express the protein of interest.

3. Results

3.1 Construction of *V. parahaemolyticus* library : DNA of *V. parahaemolyticus* ATCC 10136 was purified and used to construct a *V. parahaemolyticus* genomic library in Lambda (λ) ZAPII vector and the *Eco*RI site was chosen to construct the library. λZAPII arms were cos ligated, digested with *Eco*RI, and dephosphorelated. Ligations were packaged into λ head and plated on *E. coli* XL1-Blue. Insertion of the cloned DNA into the polylinker region within the *lacZ* gene resulted in white plaques after IPTG induction. In this experiment, the efficiency of cloning was a ratio of blue:white colonies of 3:10 which yielded 6.4 x 10^6 plaque forming units (pfu)/μg of λarms.

3.2 Immunoscreening : Recombinant λ phages which contained cloned DNA were screened with rabbit polyclonal antiserum raised against *V. parahaemolyticus* flagella. It had been decided to first use polyclonal antiserum to identify recombinant clones because the broad spectrum of reactivity may increase the chance to recognize clones carrying the gene of interest. Various dilutions of antiserum were tested with purified flagella by dot blotting to determine the appropriate dilution producing an intense reaction with the flagella, and no background. The dilutions of 1:3200 to 1:6400 were selected and used. Eight positive plaques were detected from among 60,000 plaques. Each positive plaque was immunoscreened a second time with the same polyclonal antiserum. Next, each plaque was screened with anti-core monoclonal antibody (MAb-flc) by using MAb-flc tissue culture supernatant fluid at a dilution of 1:50 to 1:100. All positive plaques tested with the polyclonal antibody also reacted with MAb-flc.

Then the pBluescript (pBS) phagemid which contained the cloned insert was rescued from the recombinant λ vector and plated on LB-Amp plates. The recombinant phagemid was purified and digested with *Eco*RI. The size of the insert, shown in Figure 1, was determined to be approximately 6.5 kb.

3.3 Transposon mutagenesis : Further investigations were carried out using transposon mutagenesis with mini-Mu dI (*lacZ*, Km^r) to define the flagellar protein coding region. *E. coli* strain POI1681 was used to provide the mini-Mu transposon. First, the recombinant pBS was purified and several attempts were made to transform the plasmid into *E. coli* POI1681 by the conventional protocol, but no transformants were obtained. Plasmid pUC 18 was used in concert as a control and the efficiency of transformation was as low as 1.3 x 10^3 cfu/μg DNA. As a consequence, electroporation was used to introduce the recombinant plasmid into the POI1681 strain. Competent cells were used at 10^8 and 10^{10} cells/ml, however, initial findings showed none or very few transformants when 10^8 competent cells were used. By contrast, when 10^{10} cells/ml were used, higher yields of transformants were obtained. For example, approximately 10^7 to 10^8 cfu/μg DNA were obtained from pUC 18, while the recombinant pBS yielded 10^4 to 10^5cfu/μg DNA. *Eco*RI digestion was done on the purified plasmids from transformants to verify that the recombinant plasmid had been introduced into

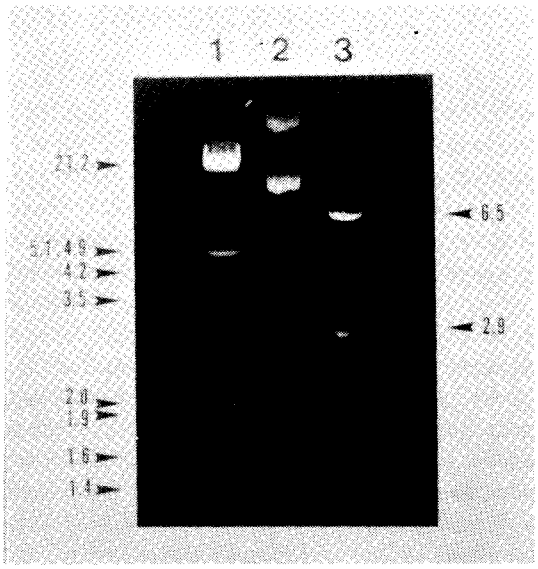

Figure 1. ***Eco*RI digestion pattern of the recombinant plasmid generated a 6.5-kb *V. parahaemolyticus* DNA insert and 2.9-kb pBS vector (lane 3) compared to undigested DNA (lane 2) and Lambda *Eco*RI-*Hind*III standard markers (lane 1).**

the *E. coli* strain.

The resulting transformants Amp^r (plasmid-encoding) and Kan^r (mini-Mu-encoding) were temperature-induced to produce a Mu phage-transducing lysate which contained recombinant plasmid pBS::mini-Mu molecules. It was noticed that lytic growth of mini-Mu in *E. coli* POI1681 was not as efficient as reported earlier when the temperature was shifted [12]. In this experiment, the lysis of temperature-induced cell culture was not observed until 2 to 3 h after the induction when examined by absorbance at 600 nm. The lysate was used to infect a Rec^+ Mu *c*ts strain (MH3497). Before the transductants were immunoscreened with MAb-flc, the XL1-Blue carrying the recombinant plasmid was induced by IPTG for expression of flagellar core protein and to determine the orientation of the flagellar gene. The result are presented in Figure 2.(Coomassie blue stain) and Figure 3. (Western blot). At time 0, there was no detectable band which corresponded to the purified flagellar core protein. Following addition of the IPTG, bands were observed at approximately 40 kD which reacted with the MAb-flc (Figure 3). These bands were detected at 1, 2, 3, 4 h and overnight culture following IPTG induction. This findings suggested that the orientation of the plasmid-encoding gene was in the same direction as the *lacZ* gene, and since it was inducible with IPTG, it requires the *lac* promoter in pBS vector for the expression. The recombinant plasmid, without mini-Mu, was also transformed into the MH3497 strain and induced with IPTG. The findings are shown in Figure 4. In contrast to the findings for the XL1-Blue strain, the bands of plasmid-encoding protein observed in MH3497 strain, were detectable before IPTG induction. The explanaton is that the lac promoter in *E. coli* XL1-Blue was effectively repressed by $lacI^q$ and requires an inducer to be able to initiate the transcription. However, the MH3497 strain produced a normal level of repressors which were not sufficient to fully repress the *lacZ* gene. Therefore, without the inducer, the *lacZ* gene can still be transcribed as well as the flagellar gene(s).

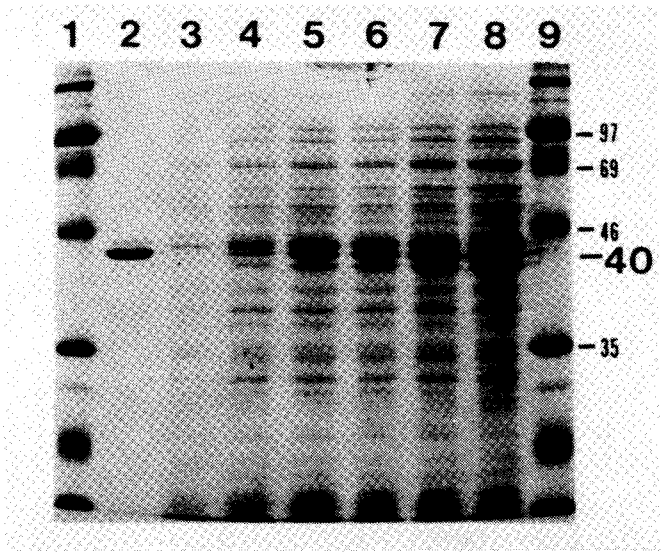

Figure 2. A pattern of IPTG induction of *E. coli* strain XL1-Blue containing recombinant pBS; before addition of IPTG (lane 3), after IPTG induction at 1 h intervals (lane 4-7) and overnight incubation (lane 8). Rainbow markers (lane 1 and 9), purified flagellar core protein (lane 2).

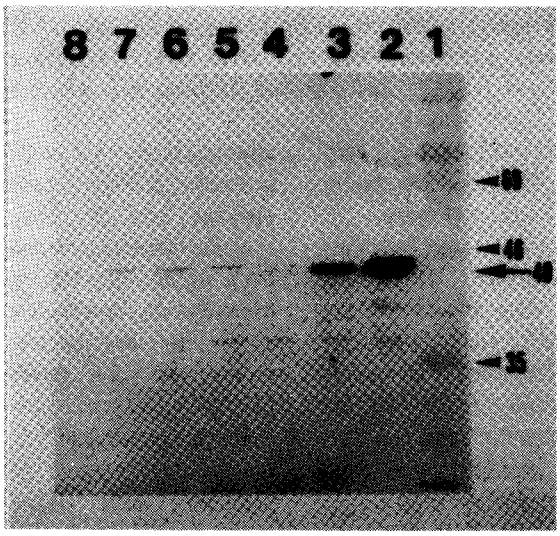

Figure 3. Western blot analysis of IPTG induced of *E. coli* strain XL1-Blue, containing recombinant pBS , with MAb-flc; before induction (lane 4), after IPTG induction (lane 5 to 8) at 1 h intervals. Rainbow markers (lane 1), purified flagellar core protein (lane 2), and *V. parahaemolyticus* cell lysate (lane 3).

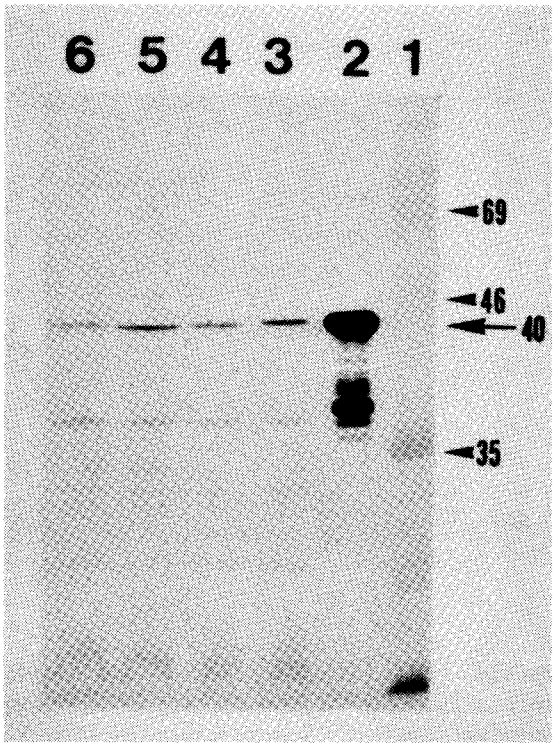

Figure 4. Western blot analysis with MAb-flc of *E. coli* MH3497 transformant, containing recombinant pBS ; before induction (lane 3), after IPTG induction (lane 4 to 6) at 2 h intervals. Rainbow markers (lane 1), and purified flagellar core protein (lane 2).

A total of 30 colonies was screened with MAb-flc by Western blot. Each were negative when exposed to MAb-flc (Figure 5). Restriction analysis yielded 6 unique locations of the mini-Mu insertion (Figure 6). Three sites designated A, B, and C, were located in the region of *lac* promoter and interupted transcription into the flagellar gene. Since mini-Mu is a large DNA segment (15.8 kb), its insertion in front of the flagellar gene can also interrupt expression of the flagellar gene located downstream. This was true for the insertion D located downstream from the *lac* promoter. Unfortunately, no positive clones (expressing flagellar protein) with MAb-flc were detected. The insertions sites, E and F, were also negative when screened with antibody. The E and F insertions were positioned in the insert fragment approximately 4.2 and 5.2 kb from the *lac* promoter. Two possibilities exists; 1) they inserted into the gene and interrupted the flagellar gene expression, or 2) they inserted in front of the flagellar gene which blocked the transcription from the *lac* promoter.

To identify the flagellar gene coding region, the first possibility was proved by generating site mutation at the *Nco*I site. This was accomplished by digestion of the recombinant plasmid with *Nco*I and filling the protuding ends with Klenow fragment to generate frameshift. The mutated plasmid was religated and transformed into XL1-Blue strain. Following induction with IPTG, a Western blot was done to compare with a positive control, the original recombinant pBS in the *E. coli* XL1-Blue strain. The results showed that the *Nco*I

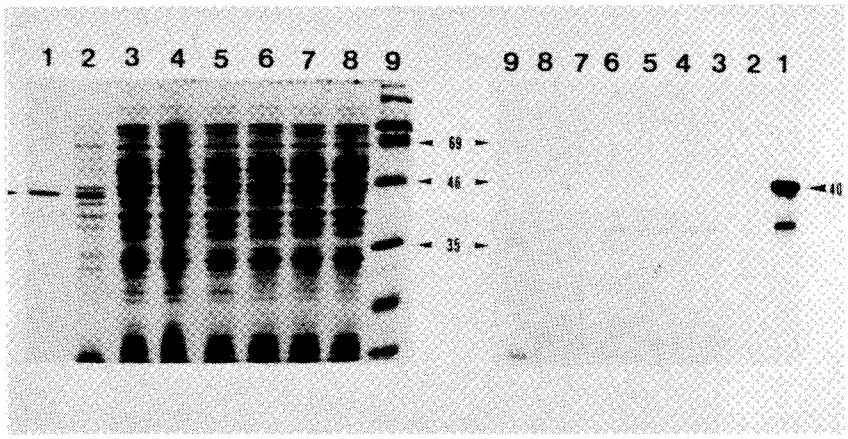

Figure 5. Western blot analyis with MAb-flc of *E. coli* MH3497 transductants containing recombinant pBS as well as mini-Mu transposon; purified flagellar core protein (lane 1), E. coli MH3497 containing recombinant pBS without mini-Mu as a positive control (lane 2), *E. coli* MH3497 transductants (lane3 to 8 for representatives A to F, respectively), rainbow markers (lane 9).

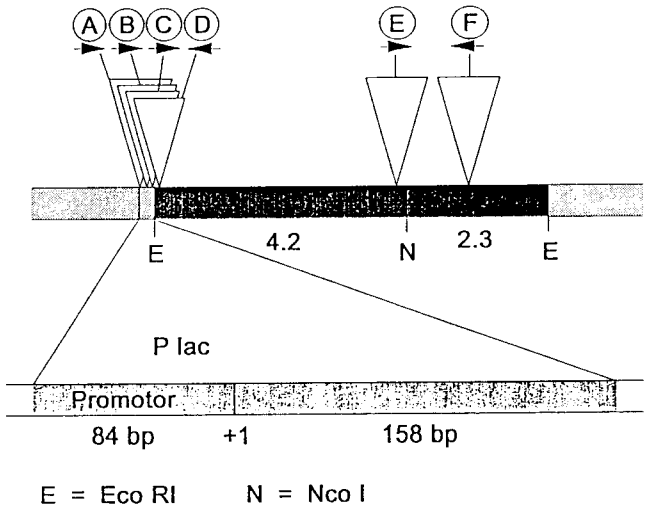

Figure 6. A pattern of Mini-Mu insertions in the recombinant pBS; A, B, and C, are located in *lac* promoter region, and D is positioned in the 6.5-kb *Eco*RI fragment just downstream from the *lac* promoter. E and F are located in the 6.5-kb *Eco*RI fragment approximately 4.2 and 5.2 kb from the *lac* promoter, respectively. Arrows indicate orientation of the mini-Mu insertions. Restriction sites are as follows: E = *Eco*RI, N = *Nco*I.

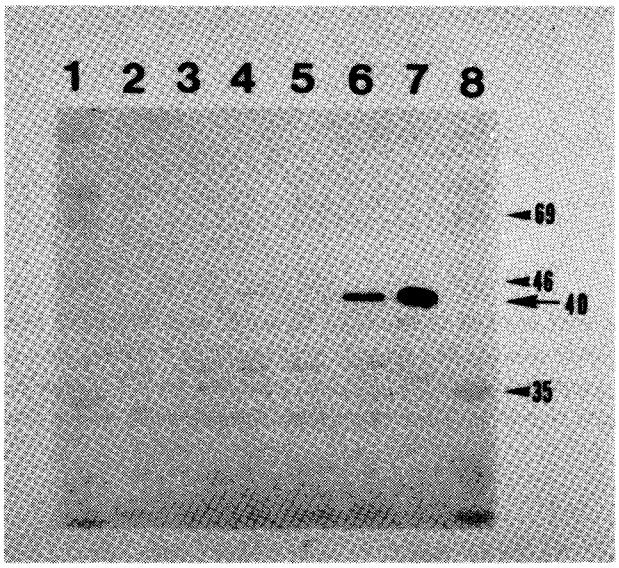

Figure 7 Western blot analysis of mutagenised *E. coli* XL1-Blue with MAb-flc; markers (lane 1 and 8), purified flagellar core protein (lane 7), *V. parahaemolyticus* lysate (lane 6), XL1-Blue with recombinant pBS before (lane5) and after (lane4)IPTG induction, XL1-Blue with *Nco*I site mutation before (lane3) and after (lane2) IPTG induction.

site mutation resulted in no expression of the flagellar gene (Figure 7). According to the molecular weight (approximately 40 kD) for the flagellar core protein, the flagellar gene is approximately 1.1 kb and the distance between E and F insertions was 1 kb. Therefore the coding region of the polar flagellar core protein should reside in *Eco*RI fragment of *V. parahaemolyticus* DNA and was located approximately 1.1 kb fragment encompassing E and F insertion sites.

4. Discussion

A *V. parahaemolyticus* genomic DNA library was constructed by using *Eco*RI in order to isolate the gene which corresponds to the species-specific polar flagellar core protein of this organism. To identify the flagellar gene coding region, Mini-Mu (15.8 kb) mutagenesis was carried out resulting in a mutant with null phenotype as well as the insertion of a large segment of DNA encoding a selectable drug resistance marker into the target gene. This large insertion of the transposon DNA allows precise physical mapping of the location of the mutation.

The result showed that the flagellar gene was encoded in the 6.5-kb *Eco*RI of cloned DNA as the protein product reacted with MAb-flc. Restriction analysis with *Eco*RI and *Nco*I indicated one *Nco*I site approximately 4.2 kb from 3'end of the *lac* promoter. *Nco*I site mutagenesis disrupted the functional flagellar gene in the insert. According to the mini-Mu insertion map, it was suggested that the flagellar gene encompasses the mini-Mu insertions at E and F locations. However, it is unlikely that the cloned DNA contains its own promoter of the flagella gene since the expression of this plasmid-encoding gene utilizes the *lac* promoter as it was inducible by IPTG in *lac*$^-$ *E. coli* strain. Further investigation should be carried out to ensure that the flagellar gene encompasses E and F locations. To do so, the 4.2 fragment has to be removed which should result in blockage of the flagellar gene expression.

5. References

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