Laboratory Exercise

Design of a Comprehensive Biochemistry and Molecular Biology Experiment: Phase Variation Caused by Recombinational Regulation of Bacterial Gene Expression

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Abstract

Scientific experiments are indispensable parts of Biochemistry and Molecular Biology. In this study, a comprehensive Biochemistry and Molecular Biology experiment about *Salmonella enterica* serovar Typhi Flagellar phase variation has been designed. It consisted of three parts, namely, inducement of bacterial Flagellar phase variation, antibody agglutination test, and PCR analysis. Phase variation was observed by baterial motility assay and identified by antibody agglutination test and PCR analysis. This comprehensive experiment can be performed to help students improve their ability to use the knowledge acquired in Biochemistry and Molecular Biology. © 2014 by The International Union of Biochemistry and Molecular Biology, 42(3):224–229, 2014.

Keywords: *comprehensive experiment; Salmonella enterica serovar Typhi; phase variation; recombinational regulation of gene expression*

Biochemistry and Molecular Biology describes in molecular terms the structures, mechanisms, and processes shared by all organisms and provides organizing principles that underlie life in all its diverse forms by using the methods of Chemistry [1, 2]. Scientific experiments are indispensable parts of this subject. The Contents of Biochemistry and Molecular Biology experiments keep changing as new technologies are being invented leading to new discoveries. New experiments ought to be developed to keep pace with the new findings. These experiments should be designed in such a way as to capture the students' interest as well as impacting knowledge about principles and facts of science in general. The design should also aim at building up the students' motivation for active participation in the study of Biochemistry and Molecular Biology in particular. Many reformations about the experiment contents have been reported [3–6]. They focus on improving the problem analytical skills, problem-solving, and innovative capabilities of students.

Salmonella, which inhabits the mammalian intestine, moves by rotating the flagella on its cell surface. The many copies of the protein flagellin, which make up the flagella, are prominent targets of mammalian immune systems [7, 8]. *Salmonella* can however evade the immune response by switching between two distinct flagellin proteins (FljB and FliC) using a process called phase variation [9–11]. Most *Salmonella enterica* serovars have two distinct flagellin genes, *fliC* and *fljB*, located at chromosome 43 and 56, respectively. Their products are different flagellins (FljB and FliC) [10]. FljA, the product of *fljA* is expressed along with *fljB*, the repressor protein that represses transcription of the *fliC* gene. The phase variation is accomplished by inversion of a segment of DNA containing the *fljB* promoter. The inversion is catalyzed by a recombinase encoded by *hin* gene. The recombination sites (inverted repeats) are called *hix*. When the DNA segment is in one orientation, the genes for FljB flagellin and the repressor FljA are expressed; the repressor FljA shuts down expression of the gene for FliC flagellin. When the DNA segment is inverted, the *fljA* and *fljB* genes are no longer transcribed, and the *fliC* gene is induced as the repressor becomes depleted.

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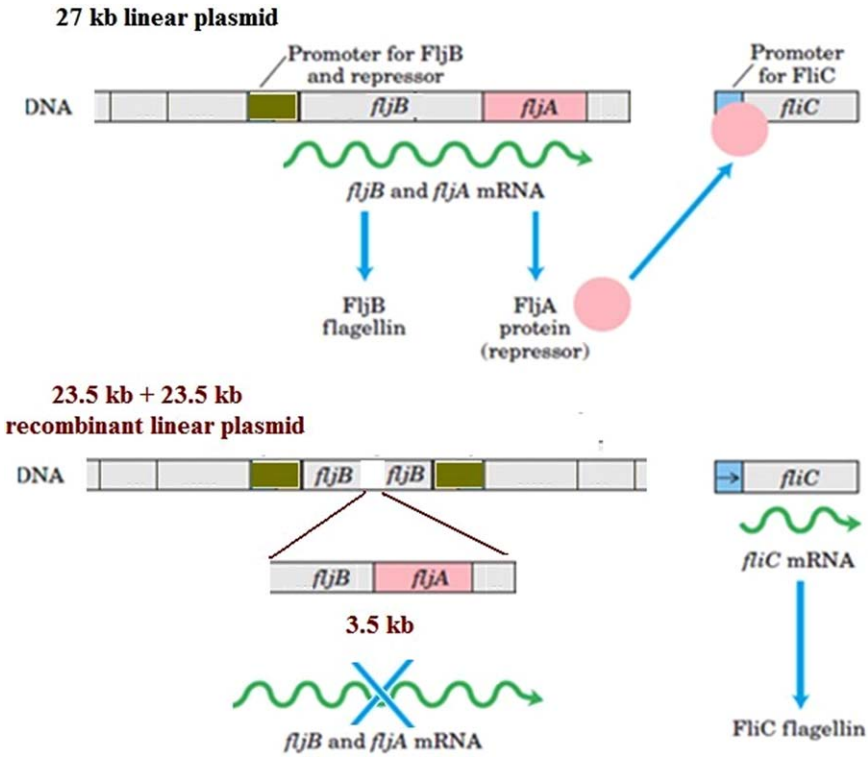

Fig 1 *Phase variation mechanism in S. Typhi, S. Typhi is a z66 antigen–positive strain, genes for FljB and FljA are expressed; the repressor FljA shuts down expression of FliC flagellin. After inducement by anti-z66 antiserum, pBSSB1 experiences a deletion of a 3.5-kb terminal region containing majority of the fljBA gene cluster and two incomplete pBSSB1 form a new 47-kb linear plasmid. The fljA and fljB genes are no longer transcribed, and the fliC gene is induced. However, when treated with anti-d/j antiserum, the d/j antigen cannot revert to z66 antigen because of lack of fljBA gene cluster.*

The Hin recombinase, encoded by *hin* in the DNA segment, is expressed when the DNA segment is in either orientation, so the cell can always switch from one state to the other, and only one of the two flagellin genes is expressed at any given time [11]. This type of phase variation mechanism is a typical gene regulation by DNA rearrangements that move genes and/or promoters and has already been demonstrated as an example of genes regulated by genetic recombination in the famous teaching material Lehninger Biochemistry 4th edition [1].

z66 antigen–positive *S. enterica* serovar Typhi (*S*. Typhi) is a special biphasic *Salmonella* whose phase 2 flagellin gene *fljB* and the *fljA* is located in a linear plasmid named pBSSB1 [12–14]. It can perform unidirectional flagellar phase variation, only switch from z66 to d/j antigen after inducement by an anti-z66 antiserum, whereas the d/j-positive strain cannot revert to z66 upon treatment with anti-d/j antisera [15, 16]. Z66 antigen is encoded by *fljB,* whereas d antigen is encoded by *fliC*, and j antigen is encoded by a *fliC* gene that lacks 261 bp in the central variable region of the *fliC* encoding d antigen. After inducement by anti-z66 antiserum, pBSSB1 experiences a deletion of a 3.5-kb terminal region containing majority of the *fljBA* gene cluster and two incomplete pBSSB1 form a new 47 kb linear plasmid. Thus, FljA and FljB are no longer expressed, and the *fliC* gene is induced. However, when treated with anti-d/j antiserum, d/j antigen cannot revert to z66 because of lack of *fljBA* gene cluster (Fig. 1) [17, 18]. The unidirectional flagellar phase variation of *S*. Typhi caused by recombinational regulation of flagellin gene expression can easily be verified by antiserum agglutination test and PCR with specific primers.

In the current research work, a new comprehensive experiment about bacterial flagellar phase variation caused by recombinational regulation of prokaryotic gene expression was set up. Three parts were involved in this experiment, that is, inducement of flagellar phase variation, antibody agglutination test, and PCR analysis. This comprehensive experiment will not only help students understand the principle of recombinational regulation of prokaryotic gene expression, but also improve their innovative abilities in Microbiology and Molecular Biology.

Experiment Design

Three assays are arranged in this experiment. They are inducement of *S*. Typhi flagellar phase variation with anti-

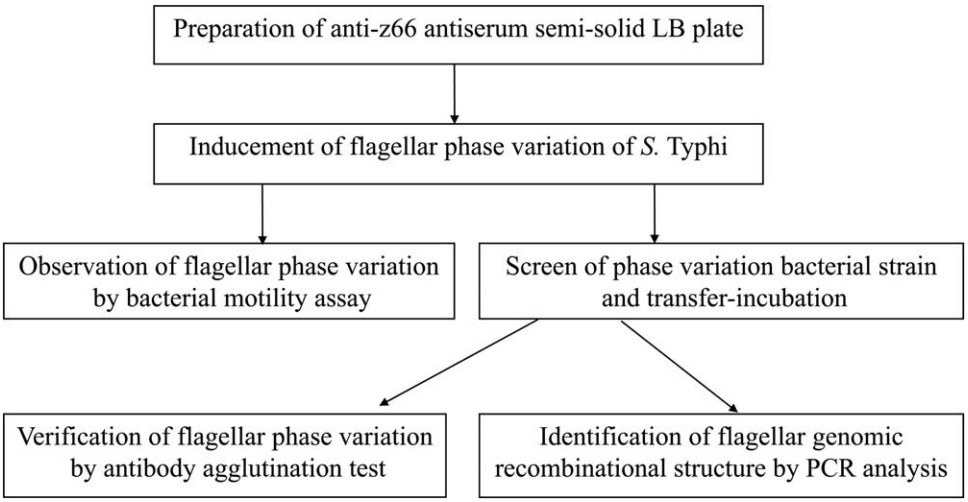

Fig 2 *Experimental procedure about regulation of S. Typhi flagellin gene expression.*

H:z66 antiserum, identification of flagellar phase variation with antiserum agglutination test, and analysis of recombination with PCR. The procedure is outlined in Fig. 2.

Materials

1. Bacterial strain: *S*. Typhi *rpoS* mutant strain, a z66-positive and low virulent strain which was constructed from the wild-type strain by using the strategy of allelic exchange mutagenesis, was used in this study [19].
2. Antibodies and reagents: Anti-z66 antiserum was previously constructed by our group [20]. Anti-d antiserum was presented by Chinese Center for Disease Control and Prevention. Other PCR reagents were purchased from TAKARA (Japan).

Methods

Preparation of Semisolid LB Plate Containing Anti-z66 Antiserum

To prepare semisolid LB plate which contains anti-z66 antiserum, 0.4 g of agar powder was added to100 mL of LB broth (pH 7.0) containing 50 mM NaCl, and autoclaved at 121°C for 20 min. The LB broth was allowed to cool on ice to a temperature of 40°C, and an anti-z66 antiserum was added to it according to the ratio of 1:300 (V_{Ab}/V_{LB}). The LB plates were stored at 4°C until use.

Inducement of S. Typhi Flagellar Phase Variation

A single *S*. Typhi z66 positive colony was inoculated at the center of the semisolid LB plate containing anti-z66 antiserum, and then incubated at 37°C for 48 hours.

Antibody Agglutination Test

Bacteria outside the original inoculation point were picked up and sub-cultured overnight at 37°C on a new ordinary LB plate to obtain single colonies. Single colonies (5–6) were randomly selected from each plate and transferred to a test tube containing 2 mL of LB and cultured with shaking at 37°C for 3 hours. The culture was centrifuged at 4,000 rpm for 10 minutes to collect the bacteria. The collected bacteria were used to perform the anti-z66 antibody and anti-d antibody agglutination test to verify the flagellar phase variation.

Flagellar Gene Recombination Analysis by PCR

fljBA gene cluster specific primers PA (TTGAAAGACA CAGGTAAGCC), PB (AGGCGTTGAGTAATTTCAGC), and PC (GCCTTGACCCAAGACTTTAG) were designed using Oligo 6.0 software. As shown in Fig 3, forward primer PA is located 48 nucleotides upstream of *fljB* start codon, whereas reverse primers PB and PC bind to sequences 710 and 340 nucleotides downstream of *fljB* start codon, located inside and outside of the deletion region of *fljBA*, respectively. Primers PA and PB produce a 777 bp amplicon in a

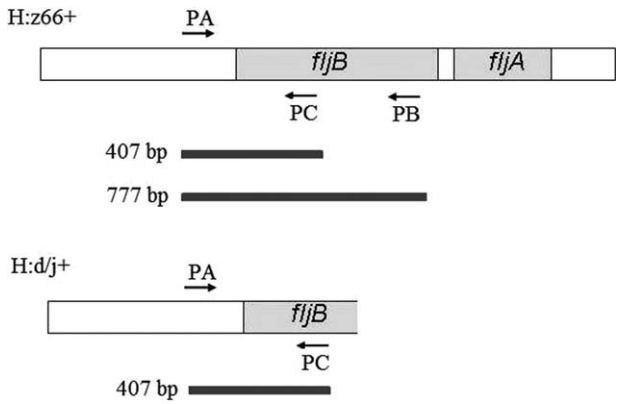

Fig 3 *Primers location in the wild-type and the phase variation strain of S. Typhi.*

PCR reaction, whereas primers PA and PC give a product 407 bp in size. Bacteria which successfully experienced flagellar phase variation (confirmed by Antibody agglutination test) were selected and cultured on an enriched LB plate. A loop full of bacteria was picked and suspended in 1 mL of autoclaved double-distilled water contained in a test tube. This tube was placed in a boiling water bath at 100°C for 10 minutes and then centrifuged at 12,000 rpm for 10 minutes. The supernatant containing such phase variation bacteria genomic DNA was used as the template for PCR. Twenty microliters (20 μL) of PCR reaction mixture, including 2.0 μL of 10× Extaq Buffer, 1.6 μL of 2.5 mmol/L dNTP, 1.0 μL of Template DNA, 1.0 μL of 5.0 μmol/L forward primer, 1.0 μL of 5.0 μmol/L reverse primer, and 0.05 μL of ExTaq DNA polymerase, was predenatured at 95°C for 5 minutes. Then, 30 cycles of 92°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute were performed with a final extension at 72°C for 10 minutes. Each PCR product was verified by agarose gel electrophoresis, and the results were observed by UV transillumination.

Experiment Observations and Results

S. Typhi Flagellar Phase Variation Induced by anti-H:z66 Antiserum

Although the flagellin gene *fljB* was expressed, *S*. Typhi *rpoS* mutant, z66 positive strain could not swim on the semisolid LB plate containing anti-z66 antiserum. In the first 24 hours, no obvious bacterial motility was observed. However, after culturing for 48 hours, bacterial circle were detected (Fig. 4), which demonstrates that the bacteria has obtained motility, and also indicates that the Flagellar phase variation had occurred.

Fig 4 *Motility of S. Typhi suggested that flagellar phase variation induced by anti-z66 antibody had occurred. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

Verification of Flagellar Phase Variation by Antibody Agglutination Test

Bacteria outside the original inoculation point were picked up and inoculated on a new LB plate for separate culture. Five to six single colonies were randomly selected on an enrichment media for antibody agglutination test. The results show that all the bacteria colonies were H:d antigen positive and H:z66 antigen negative (Fig. 5), whereas the control bacteria which were cultured on ordinary LB plate without anti-z66 antiserum were all H:d antigen negative and H:z66 antigen positive. This observation suggests that all the bacteria that were subcultured on an ordinary LB plate had experienced flagellar phase variation.

Identification of Flagellin Genomic Recombinational Structure by PCR Analysis

Gene-specific forward primer PA was designed at the upstream of *fljB* promoter region, and the reverse primers PB were located in the coding region of *fljB*. Theoretically, corresponding DNA products can be amplified in a z66-positive strain, whereas such products cannot be obtained from the phase variation bacteria due to recombination, which causes the primers cannot bind to specific template for PCR amplification. As shown in Fig. 6, when amplification was performed in z66 positive strain before inducement, a 777 bp DNA product was obtained. This product was absent in bacteria that have experienced phase variation. A control reaction was performed with primers PA and PC such that occurrence of recombination did not influence the amplification of target sequences in both H:z66 and H:d antigen positive strains. These results indicate that the structure of *fljBA* of the phase variation bacteria changes when treated with anti-z66 antibody.

Experiment Notes and Discussion

In this study, based on the principle of gene expression regulation of *S*. Typhi flagellar phase variation, a comprehensive experiment was designed specifically for medical students. Eight (8) credit hours were used to perform this experiment, and opening model was adopted. It was divided into four sections: 1 credit hour for inducement culture, 1 credit hour for subculture, 2 credit hours for

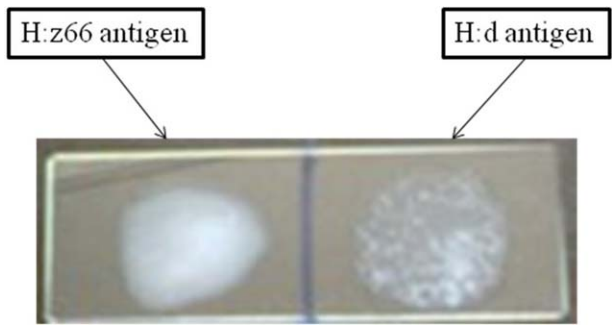

Fig 5 *Transfer-cultured S. Typhi appeared H:d antigen positive and H:z66 antigen negative, which verified flagellar phase variation had occurred.*

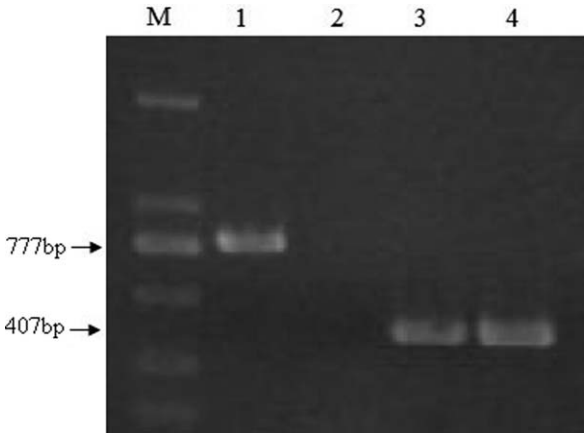

Fig 6 *PCR analysis of flagellar gene fljBA in S. Typhi phase variation bacteria. M: marker; 1: H:z66 positive strain before inducement; 2: H:d positive strain after inducement; 3: control reaction in H:z66 positive strain before inducement; 4: control reaction in a H:d positive strain after inducement.*

enrichment culture and antibody agglutination test, and 4 credit hours for template preparation and PCR analysis. This comprehensive experiment not only cultivates the students' practical ability, but also improves their comprehension on the knowledge of gene expression regulation, especially recombinant regulation. In addition, it plays an important role in stimulating their interests on scientific research and cultivating their scientific thinking abilities.

To successfully perform this experiment among undergraduates, more attention should be paid to some key points and operating precautions. First, the bacteria strains should be low-virulent or none-virulent to guarantee the students' safety. Here, *S*. Typhi *rpoS* mutant low-virulent strain was used to avoid environmental contamination and security risks caused by sterile mis-operation. Moreover, the experiment may need approval by a campus safety committee. Second, sterile operation is essential for the students to perform bacterial inoculation, transform culture, and subculture. In addition, when PCR is performed, micropipette should be correctly used and all materials and reagents should be autoclaved to prevent cross-contamination of templates.

Furthermore, if time permits, another section which includes extraction of this linear plasmid and observation by PAGE electrophoresis, extraction of flagellin protein, and Western blot analysis can be flexibly added to this experiment to enhance its comprehensiveness.

To sum up, a comprehensive Biochemistry and Molecular Biology experiment about bacterial flagellar phase variation was introduced in this study, which enables medical students to review the corresponding knowledge learned in bacteriology and molecular biology courses. In addition, this practice is helpful in developing their operating abilities, triggering their interests in learning and exploring new knowledge, and improving their problem-analysis, problem-solving, and innovation abilities [21, 22].

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