ROLE OF *Salmonella typhimurium* PROTEIN BaeR IN ABERRANT AID EXPRESSION IN B-CELL

M.Sc. Thesis

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DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

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ROLE OF *Salmonella typhimurium* PROTEIN BaeR IN ABERRANT AID EXPRESSION IN B-CELL

A THESIS

Submitted in partial fulfillment of the

requirements for the award of the degree

of

Master of Science

By

TUHIN SARKAR

DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

INDIAN INSTITUTE OF TECHNOLOGY INDORE

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "Role of *Salmonella typhimurium* protein BaeR in aberrant AID expression in B-cell" in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from July 2018 to June 2020 under the supervision of Dr. Prashant Kodgire, Associate Professor, IIT Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Tuhin Sarkar **09/07/20**

Signature of the student with date

(TUHIN SARKAR)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

P.V. Kodgire

Signature of the Supervisor of M.Sc. thesis **(Dr. Prashant Kodgire)**

Date: 14/07/2020

TUHIN SARKAR has successfully given his M.Sc. Oral Examination held on **25th June 2020**.

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Date: **14/07/2020**

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Convener, DPGC

Date: **14/07/2020**

Signature of PSPC Member #1

Date: 14/07/2020

Signature of PSPC Member #2

Date:

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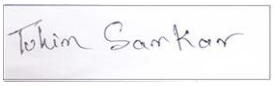

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Date: — Date: **14/07/2020**

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Lastly, I want to thank my parents and friends for supporting me and motivating me to push the limits.

DEDICATION

I dedicate this Thesis to those who have helped me immensely in my journey, my parents, my friends, my teachers and the Almighty who helped me at every step of my journey.

Abstract

Salmonella, a rod-shaped Gram-negative facultative anaerobe, is one of the most causative agents of food poisoning around the globe. It is a foodborne pathogen that is of paramount concern for public health. It gives rise to 93.8 million food-related illness and 155,000 deaths annually. One of the characteristic manifestations of *Salmonella* infection is bacteraemia. *Salmonella* BaeSR is a two-component system composed of a sensor kinase BaeS and a response regulator BaeR that binds to the DNA of the cell and regulates gene expression that functions during the stress response. Reports have shown that BaeR of BaeSR family of two-component system activates multidrug efflux, bacterial virulence activity, as well as other biological functions. Apart from these, BaeR from *Salmonella* also induces inflammatory responses including switching on of transcription factor nuclear factor-kappa B (NFκB), Janus kinase 2 pathways and the Mitogen-Activated Protein Kinases (MAPKs) thereby contribute to the inflammatory responses. Therefore, we wanted to investigate whether the *Salmonella* BaeR interacts and modulates the B-cell. And if so, then through which molecular pathway. In order to clearly know the host-pathogen interactions, we wanted to structurally characterise *Salmonella* BaeR. Our preliminary work indicated that *Salmonella* BaeR may form a dimer structure. Moreover, when B-cells were stimulated with recombinant BaeR for different time points, we observed that the expression of Activation-Indued Cytidine Deaminase (AID), the enzyme responsible for antibody diversity and specificity was significantly increased. Thus, *Salmonella* BaeR modulate B-cell biology by upregulating AID expression. Our work hints that the upregulation of AID in B-cells may be associated with different pathological conditions like B-cell lymphoma due to mistargeting of AID that can lead to mutations activating oncogenes in B-cells.

Keywords: *Salmonella,* BaeR, B-cell, Activation-Indued Cytidine Deaminase (AID).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATION

SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
AID	Activation-Induced Cytidine Deaminase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

Chapter 1

Introduction and review of literature

The Gram-negative bacteria *Salmonella*, named after Daniel Elmer Salmon was discovered in 1855 by Theobald Smith when it was isolated from the intestine of pigs infected with classical swine fever (1). *Salmonella* is one of the most infectious foodborne pathogens in the world. *Salmonella* infections occur mostly due to the consumption of contaminated food and unhygienic practices. It is a Gram-negative, rod-shaped bacterium that is a member of the family Enterobacteriaceae. *Salmonella* consists of two species: *Salmonella enterica* and *Salmonella Bongori*. *Salmonella* consists of over 2500 strains, and most of them belong *to Salmonella enterica subsp. enterica*, which is extensively accountable for *Salmonella* infections in humans (2). Invasive serotypes of *Salmonella* often result in life-threatening diseases which are cured by antibiotics but with the advent of multidrug-resistant strains, the threat of mortality due to *Salmonella* infection increases. The common manifestations of *Salmonella* infections are gastroenteritis, bacteraemia, and enteric fever (3-5). The infections majorly occur due to unhygienic practices as *Salmonella* is predominantly present in poultry animals, eggs and dairy products. Additionally, they are also present in fruits and vegetables.

Salmonella infections are more common in developing and under-developed countries due to unhygienic practices and less awareness. Mostly, South Asian and African countries are affected, giving rise to gastroenteritis, which is an inflammatory condition of the gut with symptoms including diarrhoea, nausea, headache, abdominal cramps, and myalgia. Apart from gastroenteritis, they also give rise to bacteraemia. Here, the bacteria enter the blood through the gut lining giving rise to symptoms like enteric fever and septic shock due to the activation of a severe immune response (6). Reports have shown that *Salmonella* interacts with B-cells when they enter the bloodstream. While in the bloodstream, the bacteria interact and thereby modulate the activity of the B-cells (7). Thus, the entry of *Salmonella* in the bloodstream can influence and modulate the immune system (7).

Salmonella has several virulence factors that are responsible for *Salmonella* infection and its survival in the human body. It has already been shown that BaeR is one of the virulence factors of *Salmonella*. However, not much has been investigated about BaeR. BaeR is the regulatory component of the BaeSR two-component system (8). In the current study,

we wanted to investigate whether BaeR modulates the B-cell and antibody diversity; if so, then the mechanism through which it is influencing the B-cell.

1.1 Entry of *Salmonella* into Bloodstream

One of the most common manifestations of *Salmonella* infection is bacteremia. Mostly, on-typhoidal *Salmonella* strains like *Salmonella typhimurium* are significant causes of bacteremia (3). Non-typhoidal *Salmonella* like *Salmonella typhimurium* has a broad range of hosts. Mostly in Sub Saharan African children and immunodeficient individuals, *Salmonella typhimurium* causes invasive and frequently fatal disease. *Salmonella* enters the body through fecal-oral route and pass into the intestine where they invade intestinal microfold (M) cells (9).

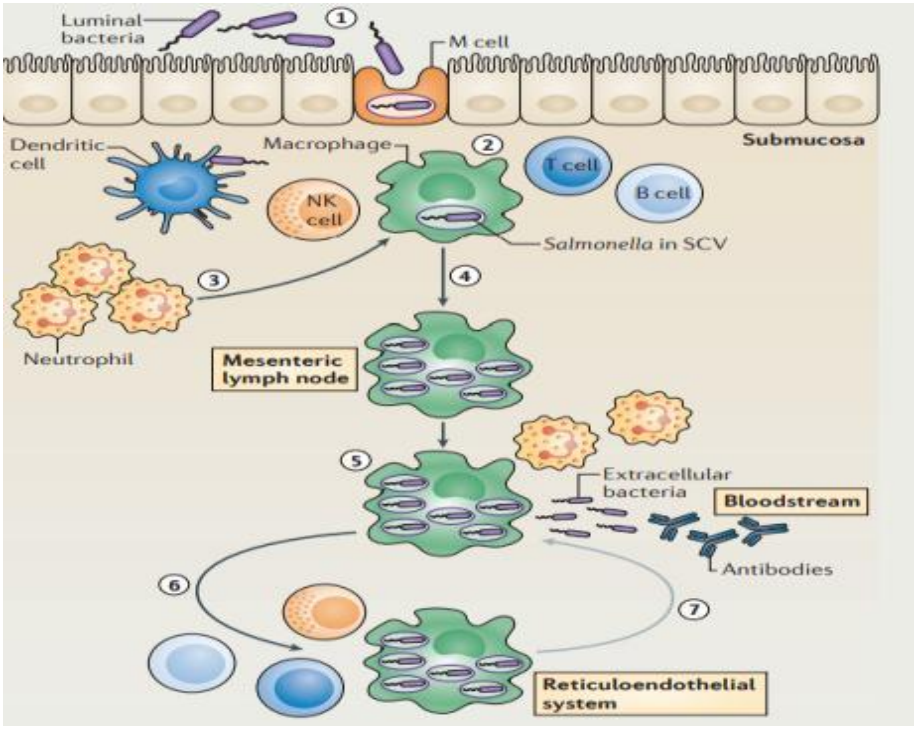


Fig. 1.1: Entry of Salmonella into the bloodstream (30)

Salmonella passes through these M cells and are grasped by the professional phagocytic cells like monocytes, macrophages, and dendritic cells in the Peyer's Patch (9). *Salmonella* infection causes the inflammation of the Peyer's patch due to the influx of the phagocytic cells at the site of infection (**Fig. 1.1**). In some cases, macrophage apoptosis can occur in a caspase1 dependent manner due to which the *Salmonella* containing macrophage may deplete, and the *Salmonella* may be released into the extracellular space (10). Another

mechanism by which *Salmonella* can enter the bloodstream is through the gut lumen within the lamina propria where it can be transported by APCs like dendritic cells (11). The spread of infection occurs through bloodstream in a systemized order as both intracellular and extracellular bacteria reproduce. Exposure of *Salmonella* in the bloodstream elicits the activation of different immune cells including B-cells and the release of several immune molecules (7). Complement mediated killing of *Salmonella* releases several pathogenic molecules into the bloodstream (12).

1.2 B-cells and *Salmonella* infection:

One of the leading players of immunity is B-cells. The B-cells are the production house of antibodies. B-cells contain BCR (B-cell receptors) that can recognize any three-dimensional structure like proteins, carbohydrates, lipids. Detection of any antigen of *Salmonella* can activate the B-cell response (13). Entry of *Salmonella* into the bloodstream activates several immune effector molecules and immune cells. Reports show that *Salmonella* interacts with many cells in the bloodstream, including NK cells, monocytes, and T-cells (14). Reports also suggest that *Salmonella* in the bloodstream interacts with the B-cells. B-cells are a major player of immunity against *Salmonella* infection. *Salmonella* virulence factors can interact with BCR response independent of T-cell and can elicit B-cell response (15). Studies suggested that B-cells differentiated into short-lived plasma B-cells that contributed to bacterial elimination in retaliation to *Salmonella* infection in a T cell-independent manner (15). Polymorph nuclear leukocytes and antibodies in the blood may detect *Salmonella* and lyse it in a complement-mediated manner, thereby releasing a number of pathogenic effector molecules in the bloodstream (10).

1.3 Role of AID in antibody diversification within B-cells

B-cells are the manufacturer of antibodies, producing different classes of antibodies. B-cells can recognize different types of antigens through B-Cell Receptors, including bound antibodies or Cluster of Differentiation molecules like CD19, CD21, and CD81. A proper B-cell response against a particular antigen involves the development of B-cells in the germinal centers, which activate the B-cell for the production of antibodies. Within the germinal center, the B-cells experience tremendous expansion. During B-cell proliferation, they

differentiate into antibody-secreting cells with simultaneous Somatic Hypermutation and class switching to produce different antibody classes with high affinity (16). Encounter with a new antigen leads to genetic alterations of immunoglobulin (Ig) genes in B-cells to produce different antibodies. The variable region of the Ig gene undergoes V(D)J recombination, followed by somatic hypermutation (SHM) to generate specificity against the antigen. Due to SHM point mutations are introduced at the variable region of Ig genes that leads to the maturation of high affinity following the antigens' final encounter. Class switch recombination (CSR) occurs with the help of AID and leads to the formation of different types of immunoglobulin classes via recombination between Sμ and the switch regions upstream of each constant region in the Ig gene locus. Antibody diversification and class switching require a critical enzyme, called Activation Induced Cytidine Deaminase (AID) (**Fig. 1.2**) (17). As the name suggests, AID deaminates deoxycytidine nucleotides to deoxyuridine nucleotides. The error-prone repair machinery then act upon the uridine nucleotides to create single-stranded breaks at the switch regions, which are converted into double-stranded breaks upon proximity and are crucial intermediates during CSR. If the single-stranded breaks are far apart, they are repaired by base excision repair (BER) enzymes and mismatch repair (MMR) pathways. Physiologically, AID is tightly regulated, and its activity is restricted to Ig genes. However, AID mis-targets non-Ig genes, including some oncogenes, resulting in oncogenic translocation and mutations that give the aberrant expression of these genes, which can lead to B-cell pathologies including B-cell lymphomas (17).

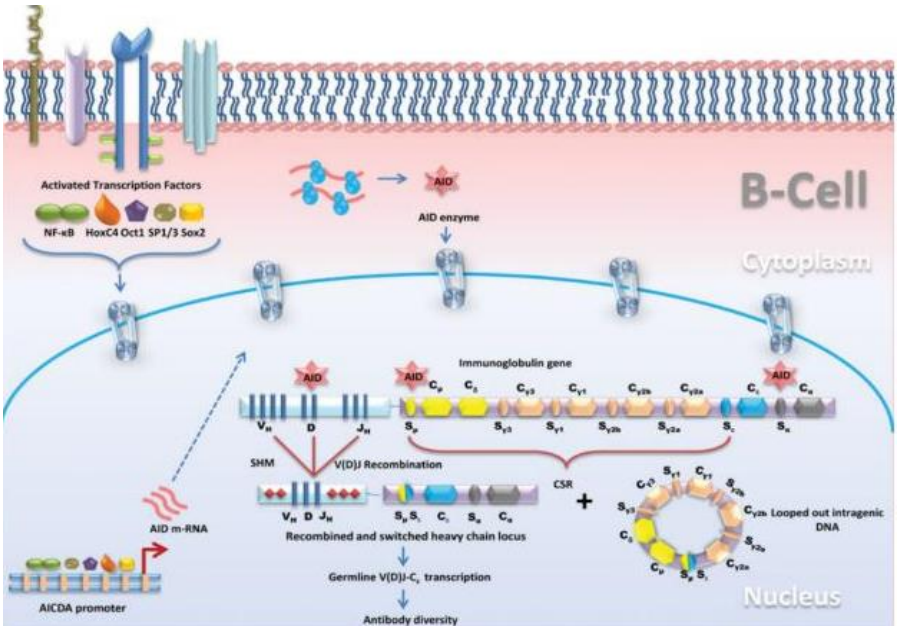


Fig. 1.2: Role of AID in Antibody diversity (17).

1.3 Regulation of AID in B-cells

As mentioned in the previous section, AID needs to be tightly controlled in order to protect other genes from having deleterious mutations and from oncogenic translocations. AID is regulated in four levels: transcriptional regulation, post-transcriptional regulation, translational regulation and via regulation of targeting. (**Fig. 1.3**) (17).

Transcriptional regulation of AID

AID gene locus has different transcription factors, enhancer, and repressor binding sites. Upstream of the AID transcription start site (TSS) are transcription factor binding sites for transcription factors like HoxC4-Oct, Sp1, NFκB, Sp3 and STAT6. Reduced AID expression occurs due to deficiency of transcription factors or blocking of the transcription factor binding sites. E-Box proteins and pax5 protein binding sites are positioned in the first intron of AID that helps in B-cell development. The AID promoter region is regulated by different factors, and dysregulation of the expression AID occurs due to a disproportionate level of the transcription factors (17).

Post-transcriptional regulation of AID

AID is regulated post-transcriptionally via microRNA-155 (miR-155) in response to external stimuli. Disorganiszation of the miR-155 binding site at 3'-untranslated region increases the AID protein, thereby increasing SHM and CSR. miR-188 also regulates AID protein level and modulate both CSR and SHM (17).

Post-translational regulation

Phosphorylation of AID is also a regulation event for its proper functioning in B-cell. Phosphorylation at serine 38 (Ser38) is thought to be important for AID activity through cAMP-dependent kinase protein kinase A (PKA). AID activity is further enhanced by interaction with replication protein A (RPA). RPA is a single strand binding protein which involved in replication, recombination and repair. Post-translational modification of AID studies have revealed that AID is also subjected to ubiquitination and regulate AID protein half-life in the nucleus (17).

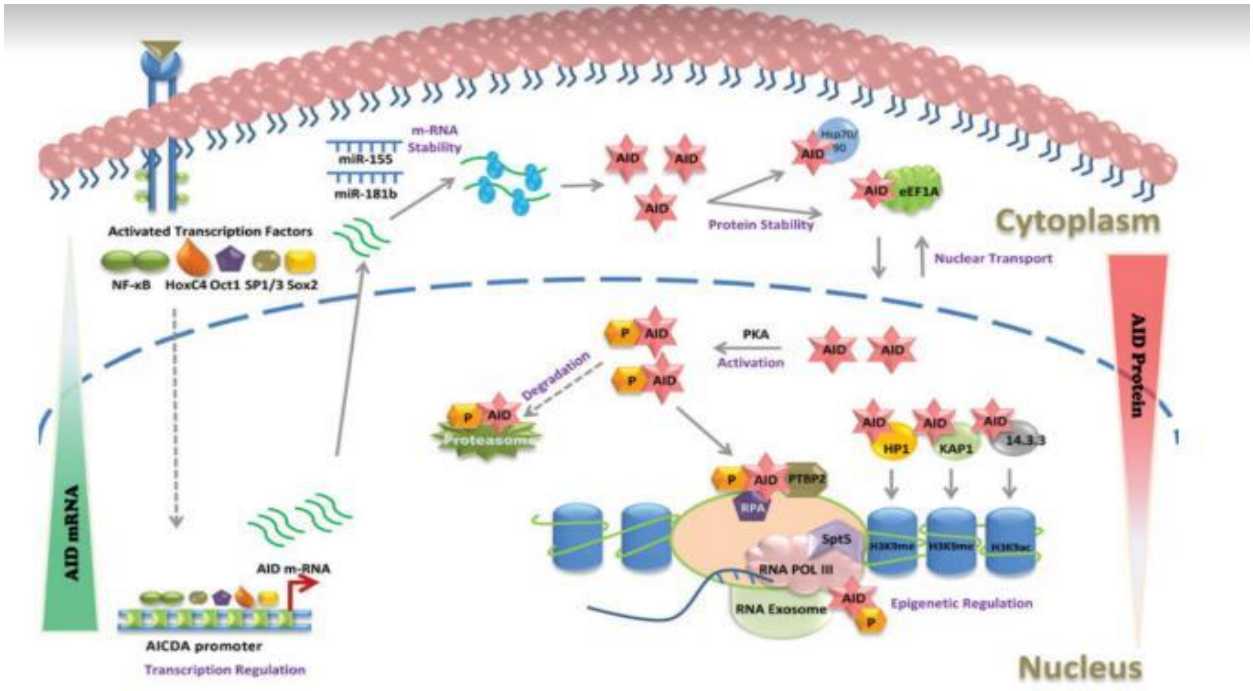


Fig. 1.3: Regulation of AID (17).

1.5 Role of *Salmonella* BaeR in *Salmonella* infection

Salmonella BaeR is a 240 amino acid containing protein that is present in the cytoplasm of the pathogen. It is a response regulator protein that is part of a two-component system named BaeSR. BaeSR two-component system consists of a sensor kinase BaeS and a response regulator BaeR that binds to the DNA of the cell and regulates gene expressions (8). Reports have shown BaeR as a virulence factor of *Salmonella*. Reports have shown its role in multidrug efflux, bacterial virulence, and other biological activities. BaeR regulates the expression of efflux pumps like AcrAB, AcrD, MdtABC (18). BaeR also activates the mitogen-activated protein kinases (MAPKs), nuclear factor kappa B (NFκB), Janus Kinase 2(JAK2)-STAT1 and thereby contributes to inflammatory responses (19-20). It also activates the COX2 pathway thereby facilitating cytokine binding and modulating immune responses.

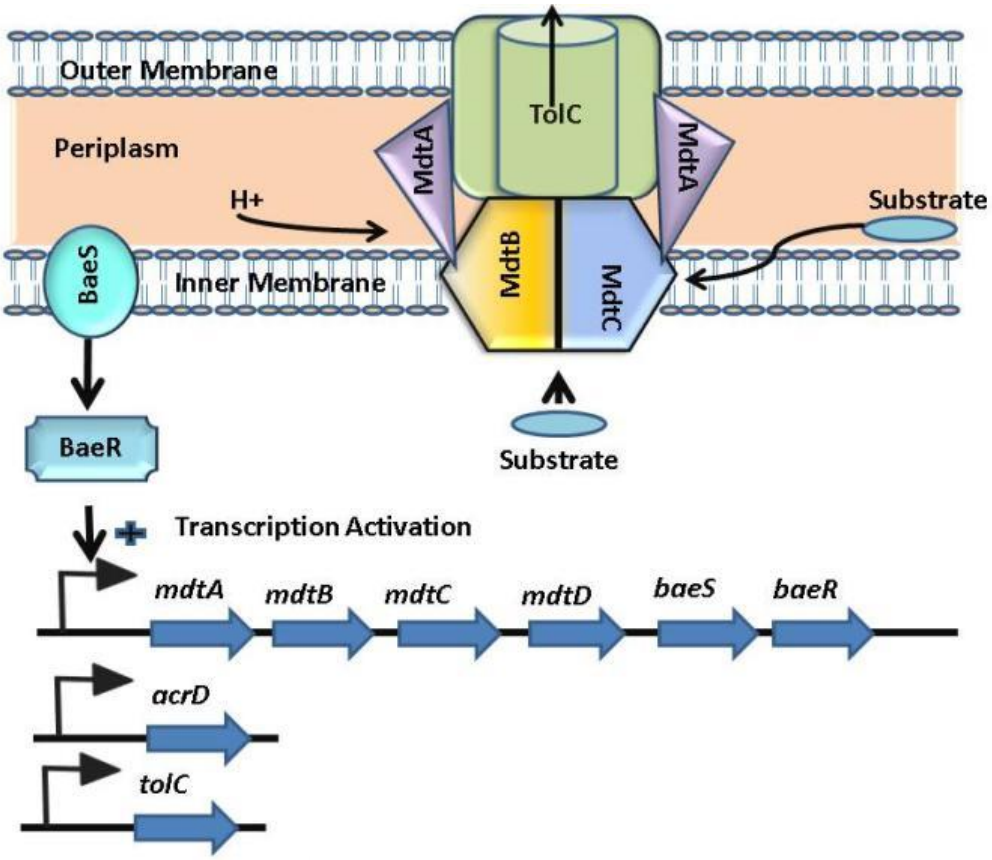


Fig. 1.4: BaeR functions as a transcription factor (18).

1.6 Thesis statement:

During *Salmonella* infection high-affinity antibodies are required for the obliteration of the *Salmonella* infection (22). It has also been shown that BaeR acts as a virulence factor of *Salmonella* and *Salmonella* modulates the activity of B-cells (20). Not much research has been done on the interaction between *Salmonella* virulence factors and B-cells. In this work, we wanted to investigate whether BaeR has any role in B-cell modulation. Through this study, we are trying to understand the host-pathogen that occurs during *Salmonella* infection in the perspective of B-cells. Not much has been studied about BaeR, and its role in *Salmonella* infection, and in this study, we aim to elucidate the role of this *Salmonella* virulence protein in modulating B-cell activity.

Characterisation of BaeR including elucidation of its structure can give us a significant view on how *Salmonella* BaeR functions. Reports of *E. coli* BaeR have already been published. In this study, we aim to study the structure and compare how similar *Salmonella* BaeR is with *E. coli* BaeR. Characterisation of *Salmonella* BaeR would give us an insight into its structural features and how closely it is related to *E. coli* BaeR whose structure has already been reported (21).

1.7 Objectives:

The objectives of the current study were as follows:

1. Cloning, expression and purification of recombinant BaeR in *E. coli*.

2. Characterisation of recombinant BaeR protein.

3. Elucidation of the functional role of BaeR in aberrant AID expression in human B-cells.

Chapter 2

2. Materials and Methods

2.1 Materials

(i) Strains: The *E. coli* strains, DH5α, and Rosetta were used as host cells for cloning and expression of BaeR. pET43 vector (Novagen) was used for cloning and expression of the protein. Human B-cells (Raji cells) were used to determine the aberrant expression of AID in response to BaeR.

(ii) Chemicals: Prep plasmid extraction mini kit, GeneJET gel extraction kit (Thermo Scientific), Agarose special low EED (HiMedia), Luria Bertani Broth Miller (HiMedia), Kanamycin (HiMedia), *Nde*I, *Xho*I, *Eco*RI (NEB). dNTPs, DNA Ligase, *Pfu* DNA Polymerase, EtBr, Lysozyme, Tris-Cl, NaCl, Imidazole, Amicon Pro Purification kit (Sigma Aldrich), Ni sepharose beads (Thermo Scientific), NiSO4, $CaCl_2$, (HiMedia) were used for cloning, expressing and purification of *baeR* gene. RPMI 1640 w/o L glutamine (Lonza), fetal bovine serum, streptomycin, penicillin, β-mercaptoethanol (Sigma-Aldrich), 100 mm sterilized cell culture plates (Thermo Scientific), RIPA buffer, protease inhibitor cocktail (HiMedia), Bradford Reagent.

2.2 Methods

2.2.1 Polymerase chain reaction (PCR)

The *baeR* gene was PCR amplified from the genome of *Salmonella typhimurium* strain 14028S using primer PK663F and PK664R (**Table 1**) by *Pfu* DNA Polymerase. The master mix was prepared using primers, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, template DNA, and *Pfu* DNA polymerase. The master mix was aliquoted into PCR tubes. The negative control was prepared similarly but without the template DNA. Program of thermocycler was set for PCR reaction with an initial denaturation at 95°C for 2 minutes which was followed by 30 cycles of denaturation at 92°C for 30 seconds, annealing at 59°C for 30 seconds and extension at 72°C for 2 minutes. At last, the final extension was set at 72°C for 10 minutes. The PCR product was analyzed using agarose gel electrophoresis.

2.2.2 Restriction Digestion of the DNA

Sequential digestion of pET43 vector and double digestion of *baeR* was done using enzyme *Nde*I and *Xho*I as per manufactures instructions at 37°C for 60 minutes, followed by heat inactivation of the enzyme at 65°C for 15 minutes.

2.2.3 Gel elution of digested DNA

The digested DNA fragments were extracted from the gel using the GeneJET purification column. The agarose gel containing the DNA fragments was excised into pieces using a clean scalpel, and the pieces were placed into the fresh weighed microcentrifuge tubes, and the weight of the gel slice was recorded, 1:1 volume of the binding buffer is added to the gel. To dissolve the gel, gels were incubated at 50°C - 60°C, and the tubes were inverted and mixed until the gel was completely dissolved. The gel solution was transferred to the column and centrifuged for 1 min. The flow-through so obtained was discarded, and the column was washed with the wash buffer by centrifuging them for 1 minute. The column was centrifuged for an additional 1minute to eradicate the residual wash buffer. The DNA was eluted in 50 µl of elution buffer and stored at -20°C.

2.2.4 *E. coli* competent cell preparation

E. coli competent cells were prepared by using $CaCl_2$ method. Briefly, cells were grown overnight in Luria Bertani broth without any antibiotic. Overnight grown bacterial culture is again inoculated for secondary culture into the LB broth in 1:100 ratio and incubated at 37 °C till the culture's OD reached 0.4-0.5 at 600 nm. All the subsequent steps were then done on the ice. Log phase cell culture was first incubated on ice for 10 minutes and then centrifuged at 4 °C for 10 minutes to pellet down the cells at 5000 rpm. Cells were then suspended in chilled $CaCl_2$ (10 ml of $CaCl_2$ for 25 ml of culture) and were incubated on ice for 45 minutes. Cells were again pellet down, and cells were gently suspended into 2 ml chilled $CaCl_2$. After 4 hours incubation on ice, cells were aliquot into pre-chilled microcentrifuge tubes with 15% pre-chilled glycerol. Cells were then stored at -80°C.

2.2.5 Ligation

Predigested DNA vector and insert with *Nde*I and *Xho*I were ligated using T4 DNA ligase (NEB) and 1x ligation buffer. The reaction mixture was incubated at 22°C for 1 hour, followed by heat inactivation at 65°C for 15 minutes.

2.2.6 Transformation of *E. coli* cells

The transformation of *E. coli* competent cells was done using the heat shock method. Competent cells were thawed on ice for some time. Subsequently, 5 µl of ligation mixture was added to the competent cells and incubated on ice for 30 min in a microcentrifuge tube. Later, cells were given a heat shock by incubating tubes at 42°C for 90 seconds followed by 120 seconds incubation on ice. 800 µl of LB broth was added to each tube and incubated for 90 minutes at 37°C. Transformed cells were then spread on to the LB agar plated containing ampicillin (100 µg/ml).

2.2.7 Agarose gel electrophoresis

DNA was separated in agarose matrix by applying electric fields. The shorter molecule travelled the larger distance in the agarose gel and the larger molecule travelled the shortest distance. Molten agarose with a fluorescent dye Ethidium bromide (EtBr) for staining the sample was poured into the casting tray of electrophoresis unit and allowed to solidify. EtBr intercalated into the rings of DNA which can then be seen under UV. The electrophoresis unit was filled with the 1X TAE buffer and the samples were mixed into the loading dye then loaded into the wells. The loading dye contained glycerol to give density to the sample and bromophenol blue to track the movement of the sample into the gel. Electric fields were then applied to separate the sample of different size from the mixture. The gel was run at 60mV for 30 minutes. After the completion of the run, the gel was then analyzed under transilluminator as EtBr intercalated DNA can be visualized under UV light as orange bands and can be distinguished according to size within the agarose gel.

2.2.8 Expression of recombinant BaeR in *E. coli*

A single colony of cells which were already transformed were taken and inoculated into LB broth containing 0.01 mg/ml Ampicillin and were grown overnight at 37°C. The secondary culture of the cells was done in fresh LB broth, and cells incubated on incubator shaker at 37°C until the OD reaches 0.8. The culture was then induced with 1 mM IPTG and incubated at 37°C for 4 hours on the incubator shaker. The cells were then collected by centrifuging the cell culture at 12000rpm for 10 minutes. The pellet obtained was then washed and suspended into the 25 mM Tris-Cl buffer (pH 7.4). To lyse the cells, 400 µg/ml lysozymes were then added to the suspended cells and incubated at 37°C for 20 minutes, followed by a sonication cycle of 3sec on 3 sec off at 65% Amplitude and centrifugation of cell lysate at 12000rpm for 10 minutes. To confirm the induction 12% SDS PAGE gel was then run.

2.2.9 Purification of the recombinant BaeR

Protein purification was done using affinity chromatography (Ni NTA column) at 4°C. The input the sample was prepared such that it had the composition as the equilibration buffer (50 mM Tris-Cl of pH 8, 500 mM NaCl, 20 mM Imidazole). The input sample was then loaded into the pre-equilibrated Ni NTA column, and the flow-through was collected. The column was then washed with Wash1 (50 mM Tris-Cl of pH 8, 500 mM NaCl, and 40 mM Imidazole) and Wash2 (50 mM Tris-Cl of pH 8, 500 mM NaCl and 80 mM Imidazole). The purified protein was eluted using 2 ml elution buffer (50 mM Tris-Cl of pH 8, 500 mM NaCl and 150 mM Imidazole).

2.2.10 Endotoxin removal from purified BaeR

Pierce endotoxin removal column was used to remove endotoxin from the purified BaeR. 0.2 N NaOH was added to the column and left overnight to activate it. Followed by activation, the column was spun at 500g for 1 min to remove the NaOH. This was followed by washing with 2N NaCl and ET free water. The resin was then Equilibrated endotoxin-free buffer. After this, the sample was added and gently mixed with the beads within the column for 1 hour. After the incubation, the flow-through was collected by spinning the column at 500g for 1 min.

2.2.11 SDS PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) was used to separate the protein on to the gel depending on their size by applying the electric field and using discontinuous polyacrylamide gel as the support medium. The smaller proteins travelled a larger distance compare to the larger protein. 12% gel was cast using glass plates, spacer, comb, casting tray, and casting stand. Samples were prepared using 1 X loading dye and heating at 95°C for 10 minutes. Samples were loaded, and the gel was run in the 1X running buffer. The gel was stained and destained for the required period of time, and the gel was analyzed.

2.2.12 Semi-native SDS PAGE

12% gel with 1% SDS instead of 10% SDS was cast using glass plates, spacer, comb, casting tray, and casting stand. Samples were prepared using 1 X loading dye with β-mercaptoethanol and 1X loading dye without β-mercaptoethanol, and a sample with β-mercaptoethanol was heated at 95°C for 10 minutes. Samples were loaded, and the gel was run in the 1X running buffer containing 0.1 g SDS/liter instead of 1g SDS/liter. The gel was stained and destained for the required period of time, and the gel was analyzed.

2.2.13 Cell culture techniques

Raji cells are immortal cell lines procured from B-lymphocytes of an 11-year-old Nigerian male patient suffering from Burkit's lymphoma. Raji cells were cultured in RPMI 1640 medium which was supplemented with 10% FBS (Invitrogen), 100 μg/ml penicillin G, and 50 μg/ ml streptomycin (Invitrogen), 1% l-glutamine (Invitrogen), and β-mercaptoethanol (Sigma-Aldrich) at 37 °C with 5% CO_2.

2.2.14 Stimulation of B-cells with recombinant BaeR

Endotoxin free BaeR was added into the 100 mm culture plate after the cells became >90% confluent in a concentration of 1 μg, 3 μg, 5 μg, and stimulated for 1 hour and 4 hours. After 1 hour, cells were collected from each plate; cells for both RNA sample and protein

samples were collected, centrifuged at 1500 g for 5 min and stored in -80°C, similarly after 4 hours of stimulation cells were stored in -80°C in order to prepare a sample for Western Blot.

2.2.15 Preparation of Western blot sample

Cells were suspended in 300 µl of 1X RIPA buffer, and 3 µl of protease cocktail inhibitor was added to it. The suspended cells were vortexed 3-5 times for 30 seconds and kept in ice after every 30 seconds. The suspension was then kept on ice for 20 min. After 20 minutes, the suspension was vortexed at 12000 g for 20 min and the supernatant was collected, and concentration was measured using Bradford assay.

2.2.16 Western Blot

Raji cells were stimulated for 1, 4 and 8 hours with 1 µg, 3 µg, 5 µg of BaeR, protein samples were prepared, and an equal concentration (80 µg) of protein samples were loaded onto a 12% polyacrylamide gel, transferred into nitrocellulose membrane, blocked with 5% skimmed milk in PBST for 3 hours. The membrane was probed with antibodies against AID, and GAPDH as a loading control, followed by washing and incubation with anti-mouse IgG-HRP secondary antibodies. The blot was visualized by enhanced chemiluminescence (ECL) system.

2.2.17 Sequence alignment using BLAST:

FASTA sequences of *E. coli* BaeR *and Salmonella typhimurium* BaeR from NCBI were used as inputs for BLAST and the program was made to run to compare and predict similarities between the two sequences.

2.2.18 Secondary Structure Prediction using SOPMA:

The Self-Optimized Prediction method With Alignment (SOPMA) is used for prediction of the secondary structure of a protein-based on the primary sequence of the protein. In SOPMA, a short homologous sequence of amino acids tends to form a related secondary structure by using template proteins present in its database. FASTA sequences of *E. coli* BaeR and *Salmonella typhimurium* BaeR from NCBI were used as inputs. After inputting the FASTA sequences, the output width was selected as 70 and the number of

conformation was selected as 4 from the drop-down box. Followed by the setting up of the parameters, the sequences were submitted individually.

2.2.19 3D Model prediction using GalaxyWEB:

GalaxyWEB server is used to predict protein structure from a sequence by template-based modeling and causes refinement of loops or terminus regions by *ab initio* modeling. FASTA sequence was submitted in the server (http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=TBM), followed by the email address and Job name. Optionally choice between oligomeric states can also be used as input. The FASTA sequences with the job name and the email address were submitted and the 3D model was obtained in the mail.

CHAPTER 3

Results and Discussions

3.1 Cloning of *salmonella baeR* with C-terminal His-tag

In our study we want to know how *Salmonella* BaeR and *E. coli* BaeR are similar in terms of structure, we also want to know the functional significance of this virulence protein with respect to B-cells. To obtain pure BaeR protein, the *baeR* gene had to be cloned into an expression vector and expressed followed by purification to obtain pure protein. We chose pET43 as an expression vector as it has 6x His tag in its C-terminal that helps in purification of recombinant protein using Ni-NTA column and *E. coli* Rosetta cells were used as the expression host. pET43 has two unique sites *Nde*I and *Xho*I which were used for the cloning the gene directionally into the plasmid (**Fig. 3.1**). So at first, *Salmonella baeR* was amplified by PCR from the *Salmonella* genome using genome-specific primers PK 663F and PK664R (**Table 1**) having *Nde*I and *Xho*I, respectively. The PCR product was digested using *Nde*I and *Xho*I restriction enzymes and ran on a 0.8% agarose gel. After successful digestion, ligation and transformation of the recombinant plasmid was performed.

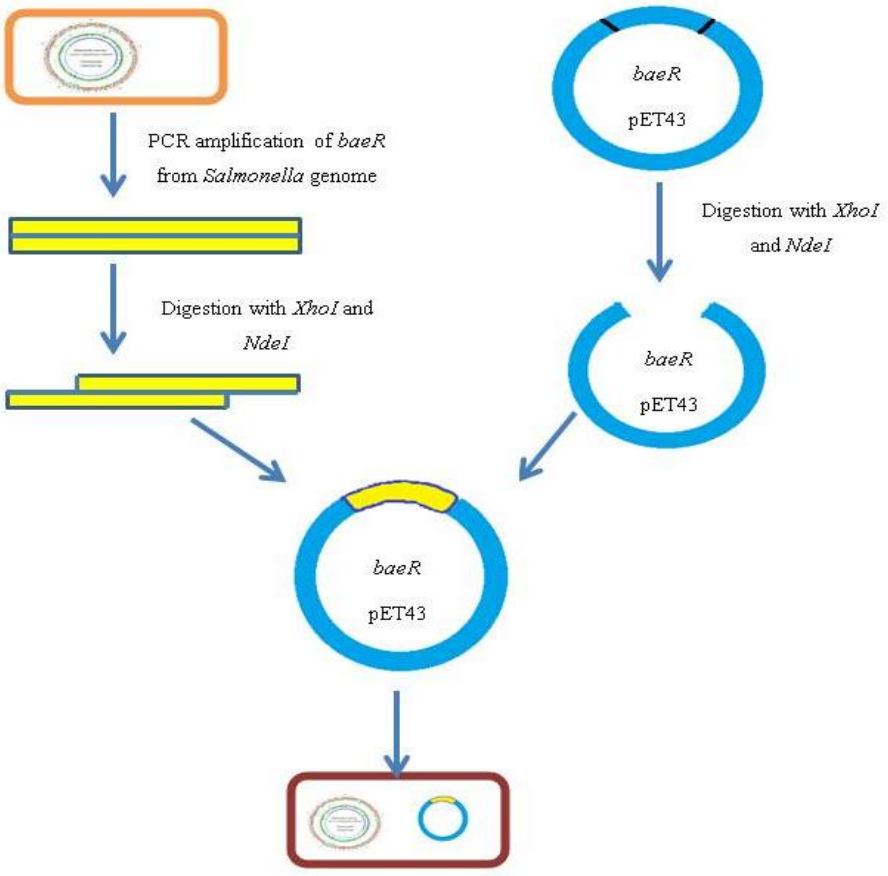


Fig. 3.1 Cloning Strategy of *baeR* in pET43 expression vector

In the gel image shown in **Fig. 3.2** the lane # 1 shows the 1kb ladder and lane # 2 shows the PCR amplified *Salmonella baeR* gene which has a size of 730 bp. The vector pET43 was also digested using *Xho*I and *Nde*I, to generate complementary sticky ends with that of the PCR amplified gene and confirmed by running the samples in 0.8 % agarose gel.

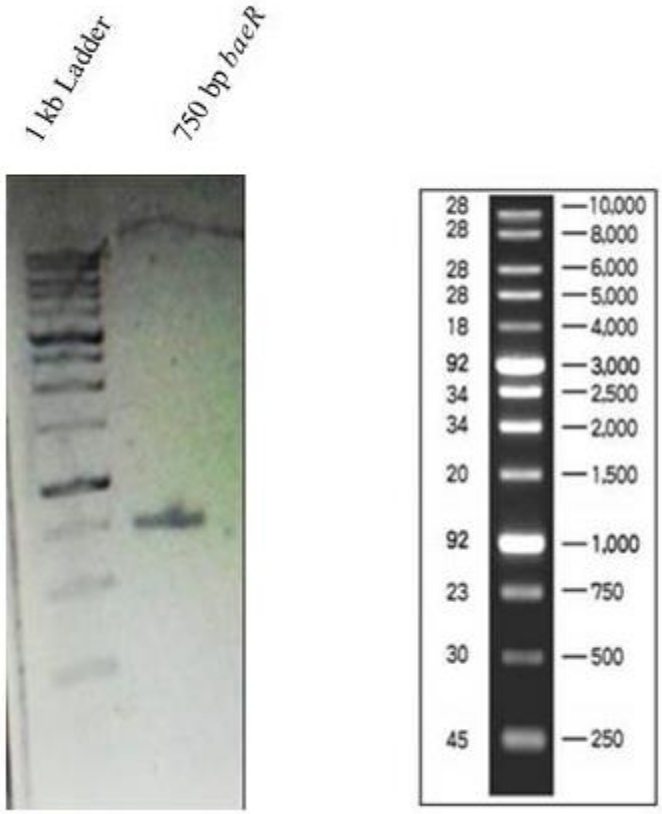


Fig. 3.2: PCR amplification of *baeR* gene from *Salmonella* genome

Fig. 3.3: Double digested insert and vector

In the gel image shown in **Fig 3.3** the lane # 1 shows the 1kb ladder, lane # 2 shows the double digested *baeR* gene and the lane # 3 shows the double digested pET43 vector. In order to clone, the digested PCR fragment into the plasmid, the double digested PCR product, and the double digested vector were ligated using T4 DNA ligase.

After ligation, transformation was needed to be performed, followed by screening via colony PCR and restriction analysis to check the transformation and subsequently sequencing to confirm the success of the transformation. Thus, in order to express the gene, the gene needs to be transformed into a favorable host for expression.

Therefore, we transformed the recombinant *baeR* containing pET43 into *E. coli* Rosetta cells as these cells lacks the gene for Lon proteases that degrade many foreign proteins, it also lacks gene coding for the outer membrane protease OmpT, that degrades extracellular protein. In order to avoid such degradation of protein, *E. coli* Rosetta cells were used.

To preliminarily confirm if the transformation has happened or not, we used colony PCR. In colony PCR, we took a colony from the transformation plate and added it to the PCR Master Mix having the primers for *baeR*. In the gel image shown in **Fig. 3.4** in the first gel image lane # 1 shows 1kb ladder, lane # 5 shows positive clone 5, lane # 10 shows positive clone 10. Similarly, in second gel image lane # 1 shows 1kb ladder and lane # 6 shows positive clone 21.

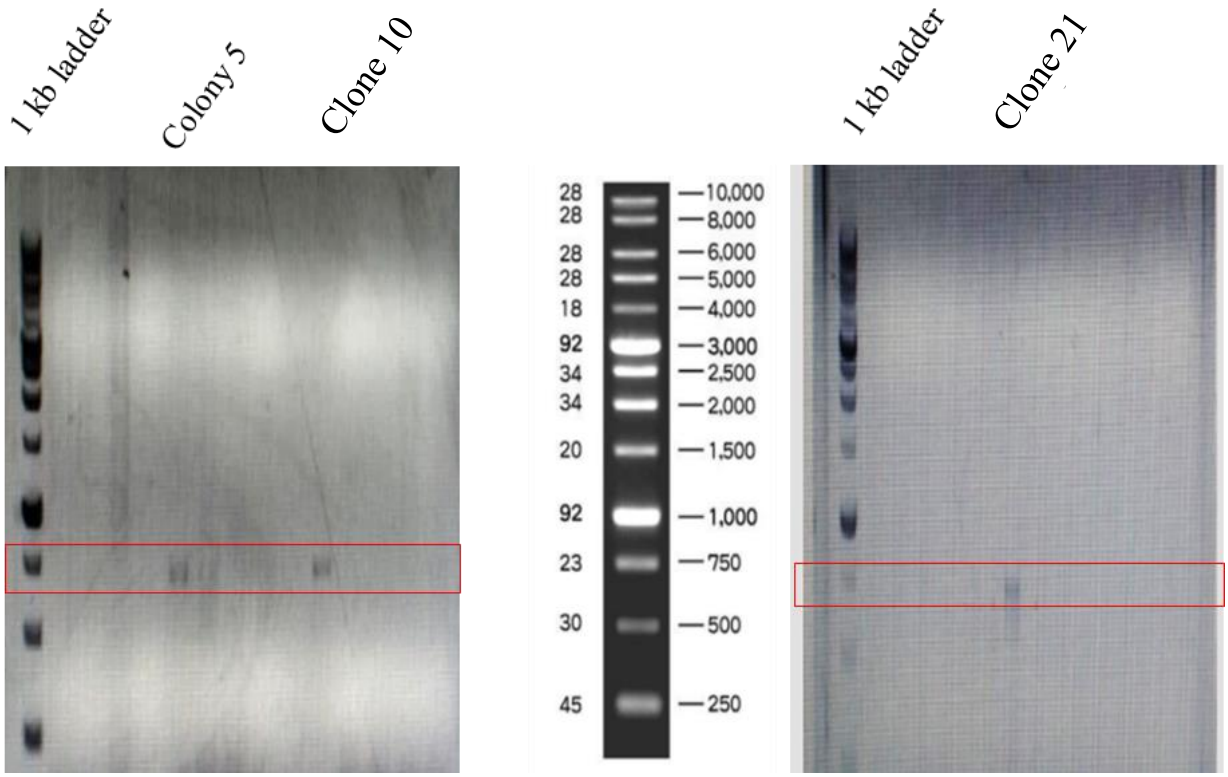


Fig. 3.4: Colony PCR for confirmation of recombinant *baeR*

To further verify, DNA from the positive colony cultures of the replica plate was isolated and restriction digested using *Eco*RI since the parent plasmid pET43 will lose its unique *Eco*RI site present in the MCS, and the PCR fragment had only one *Eco*RI site. Thus, if transformation occurred, it would give only one single band of 7.5 kb. In the gel image shown in **Fig. 3.5** the lane # 1 shows the 1kb DNA ladder, lane # 2 shows the undigested plasmid and the lane # 3 shows the *Eco*RI digested plasmid at 7.5 kb.

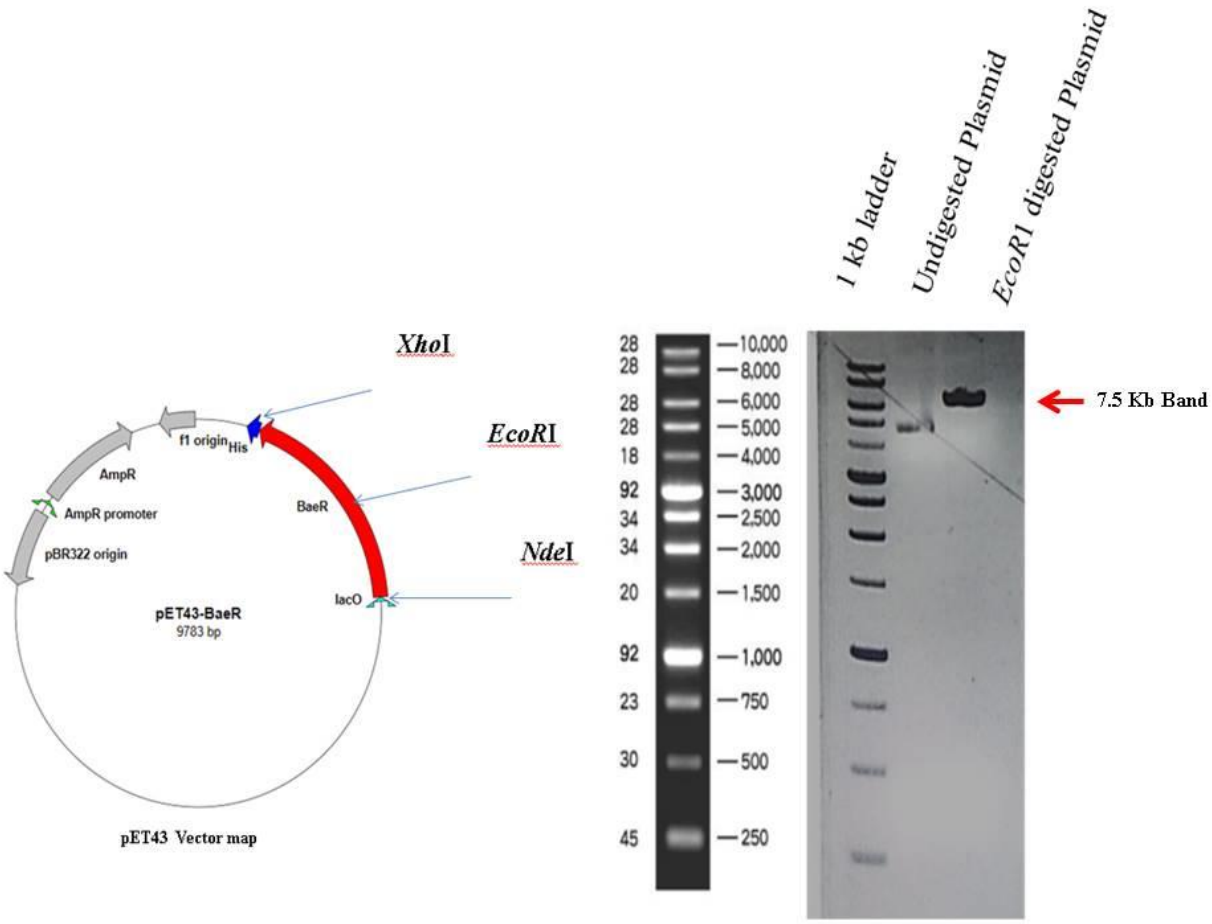


Fig. 3.5: Restriction analysis of recombinant vector for confirmation

3.2 Expression of recombinant BaeR:

BaeR is a cytoplasmic protein so to enhance the expression of the protein; the expression vector pET43 has a lac operator and promoter upstream of the MCS where *baeR* has been cloned. Thus, the transformed *E. coli* cells were induced with IPTG and grown to mid-log phase. The bacterial cells grow exponentially hence express the protein in the large amount at the mid-log phase. As BaeR is a cytoplasmic protein, the expressed protein was obtained in soluble form by lysing the cell wall of the bacteria by adding Lysozyme. Lysozyme is glycoside hydrolase, which breaks down the β (1, 4) glycosidic linkages between N-acetylmuramic acid and N-acetyl-Dglucosamine in peptidoglycan. The cells were further agitated by applying high sound energy (>20 kHz) using ultrasonicator. The protein so obtained in the soluble fraction was then confirmed by running the SDS PAGE. BaeR protein is of 27 KDa, but the His-tag at the N terminal added 1 KDa to the mol. wt of the protein, as a result, a 28 KDa band was observed confirming the presence of protein in the soluble fraction.

In the polyacrylamide gel shown in **Fig. 3.6** the lane # 1 shows protein ladder, the lane #2 shows sample containing uninduced pellet, the lane # 3 shows uninduced supernatant while lane # 4 and # 5 contains IPTG induced pellet and supernatant. Lane # 5 shows that BaeR has been induced and present in the supernatant.

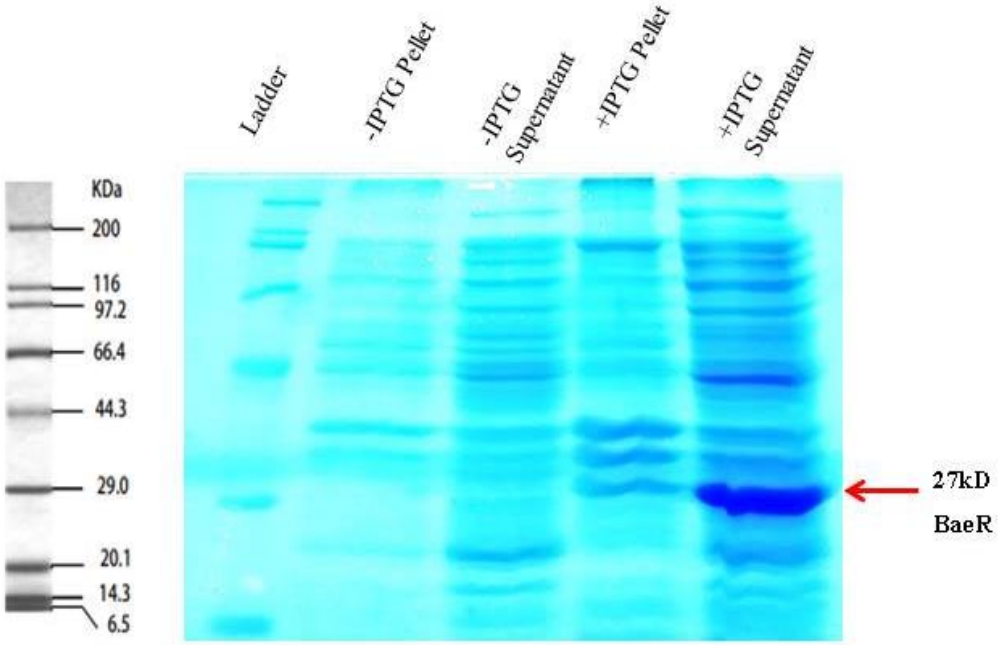


Fig. 3.6: SDS PAGE showing induction of BaeR of size 27KDa.

3.3 Purification of recombinant BaeR

Recombinant BaeR has a Poly Histidine tag containing 6 residues of histidine on its Carboxyterminal. Histidine and Nickel has a high affinity for each other. The histidine tag of the recombinant protein binds strongly to the Nickel of the Ni-NTA column. So when the lysate is passed through the column, the high-affinity recombinant protein binds strongly to the Ni-NTA agarose beads in the column, which gets eluted when imidazole in elution buffer starts competing for the binding sites in Ni-NTA column and thereby elutes out the protein. Recombinant BaeR was purified from the crude extract by affinity chromatography using Ni-NTA columns. The purity of the protein was tested by running the SDS PAGE. Purified protein was obtained in E1.

In the SDS gel image shown in **Fig. 3.7** the lane # 1 corresponds to the ladder, the lane # 2 corresponds to the input i. e. the induced supernatant, the lane # 3 corresponds to the Flowthrough obtained when the induced supernatant was passed through the Ni-NTA column, lane # 4 and # 5 are Wash 1 and Wash 2, respectively, i. e when the column was washed with wash buffers 1 and 2 respectively. Lane #6 corresponds to the fraction obtained when elution buffer 1 was applied and lane #7 is the fraction that obtained after elution buffer 2 was applied. The last lane corresponds to the fraction obtained after the stripping of the column with the stripping buffer.

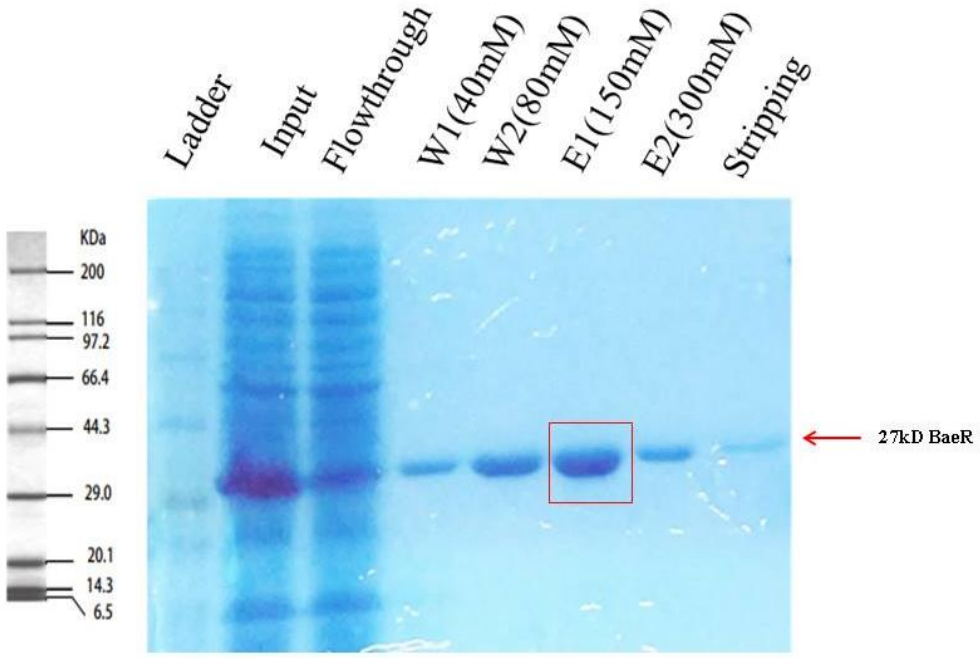


Fig. 3.7: SDS PAGE of purified BaeR.

3.4 Sequence Alignment of *E. coli* BaeR and *Salmonella* BaeR:

BaeR is present in both *E. coli* and *Salmonella*, in order to know whether the amino acid sequence is conserved among these species and if so then by how much, sequence alignment was done between the protein sequence of *Salmonella* and *E. coli*. FASTA sequence of *E. coli* BaeR and *Salmonella tyhphimurium* BaeR from NCBI were used as inputs for BLAST and the program was made to run to compare and predict similarities between the two sequences. Sequence alignment resulted in the alignment of the two sequences with an identity of 96.7% and a similarity of 98.3% differing only in 8 amino acids. The changes in amino acids are as followed: S41N, Q45K, I144M, G146S, R155C, M158A, H174L, I239L. In three positions the amino acids are substituted by an aliphatic amino acid in *Salmonella* BaeR while in position 144 and 155 the amino acids are substituted by sulfur containing amino acids and in position 45 the neutral amino acid Glutamine is replaced by basic amino acid lysine while in position 41 hydroxyl group containing Serine is replaced by neutral amino acid Asparagine.

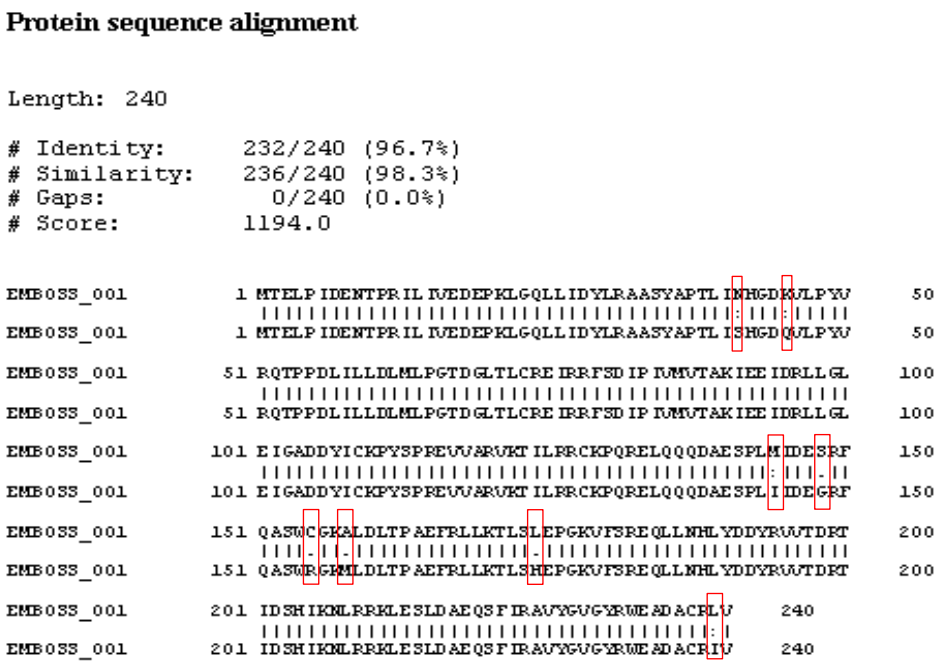


Fig. 3.8: Sequence Alignment of *Salmonella* BaeR and *E. coli* BaeR

3.5 Secondary structure comparison using SOPMA:

SOPMA was used to predict secondary structure including the percentage of random, alpha, extended, and beta coils. The secondary structure prediction indicates whether the given amino acid lies in any one of the secondary structures. Prediction using SOPMA predicts that random coils dominate BaeR followed by alpha-helix in both bacteria and there is only difference between extended strands, beta turns and bend regions. As shown in the **Fig. 3.9** the *Salmonella* BaeR contains 90 alpha-helices which are the same as *E. coli* BaeR but it contains 49 extended strands, 17 beta turns and 84 random coils while *E. coli* BaeR contains 47 extended strands, 20 beta turns and 83 random coils.

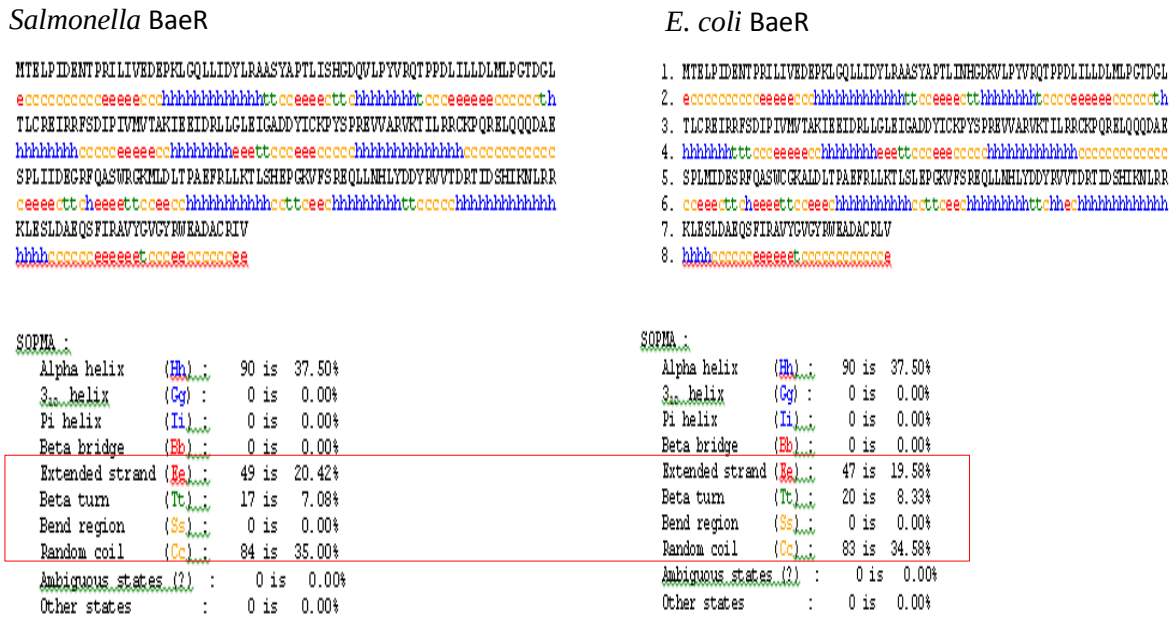


Fig. 3.9: Secondary structure comparison of *Salmonella* and *E. coli* BaeR.

3.6 Comparison of different secondary structures between *Salmonella* and *E. coli* BaeR:

In order to compare whether *Salmonella* BaeR and *E. coli* BaeR have similar secondary structures and whether the same amino acids are responsible for the same secondary structures NETSURFP 2.0 was used to generate the **Fig. 3.10** (29) which gives a graphical representation of where each amino acids were present within the secondary structures. In this figure, the amino acid residues are lined above and below each amino acids

the secondary structure which the amino acid forms are given, where the orange wave signifies the alpha helix, the purple arrow signifies the strand and pink line signifies the coil and disorder is signified by a grey line whose thickness indicates the probability of the disorder. From the **Fig. 3.10** we can see that both the structures are mostly dominated by random coils and alpha helixes with differences very negligible within extended strands and Beta turns. We can also see the probability of the disorder increases for the residues which are involved in random coils which is expected.

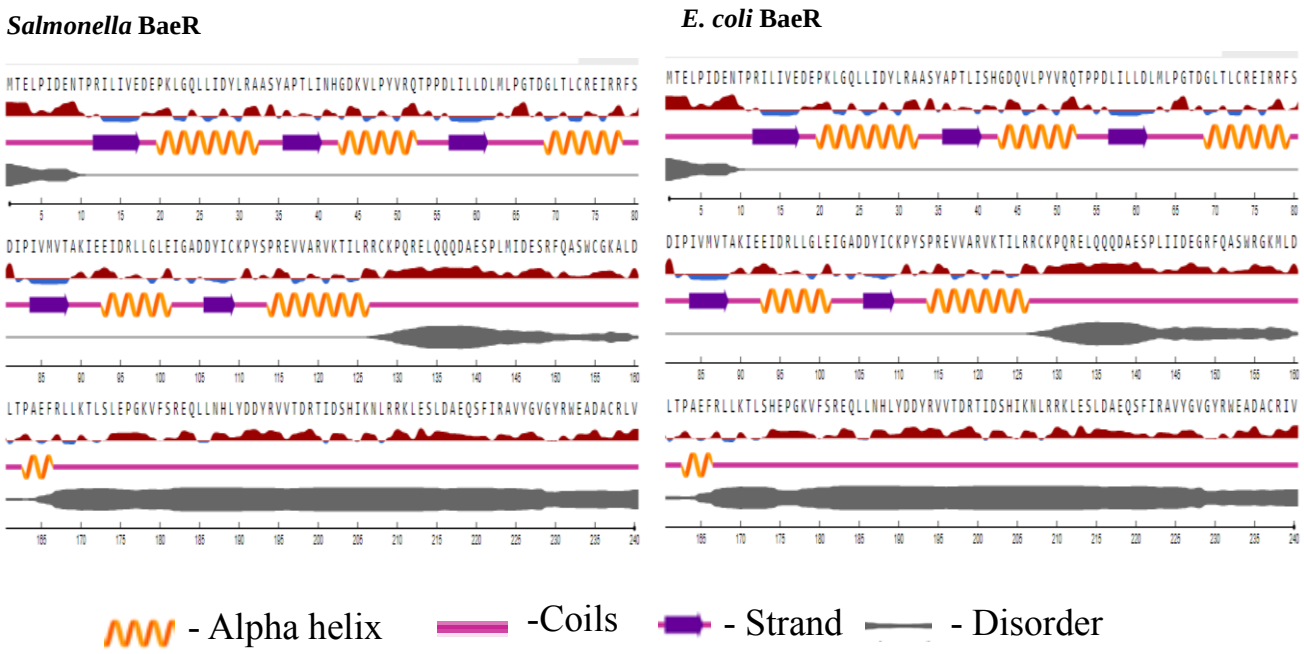


Fig. 3.10: Graphical representation of the location 240 residues within secondary structures of *Salmonella* and *E. coli* BaeR .

3.7 Dimer prediction of *Salmonella* BaeR using GalaxyWEB:

The response regulator component of the BaeSR two-component system belongs to the OmpR/PhoB family of transcription factor Response Regulators (RRs) (26). The characteristics of the response regulators of this family are that they contain an effector and a receiver domain which are linked by a flexible linker. The N-terminal receiver domain constitutes of a conserved aspartate and the C-terminal effector domain constitutes a DNA-binding domain which takes part in the transcriptional regulation of genes (26). Depending upon the phosphorylation state of the key Aspartate residue and the orientation of the switch residues, Tyr/Phe and Ser/Thr, in the receiver domain, they are active or inactive. The

phosphorylation shifts the balance towards the dimeric form that is required for binding of DNA. Phosphorylation of the Response regulators sets off dimerization at their α4-β5-α5 face of the receiver domain (21, 27). Re-adjustment of effector domains is necessary for the BaeR to bind to the DNA; the presence of the linker and the elimination of interdomain contacts would easily allow re-adjustment of the domain in the presence of DNA.

The Galaxy Homomer server predicts the monomer and the homo-dimer structure of a desired protein from a monomer structure or FASTA sequence. It performs both template-based modeling and *ab initio* docking, and also does model refinement that can persistently enhance model quality. A number of options are provided by the server that the user can choose, which depends on the information available on monomer structure, oligomeric state and locations of unreliable/flexible loops or termini. With the combination of additional

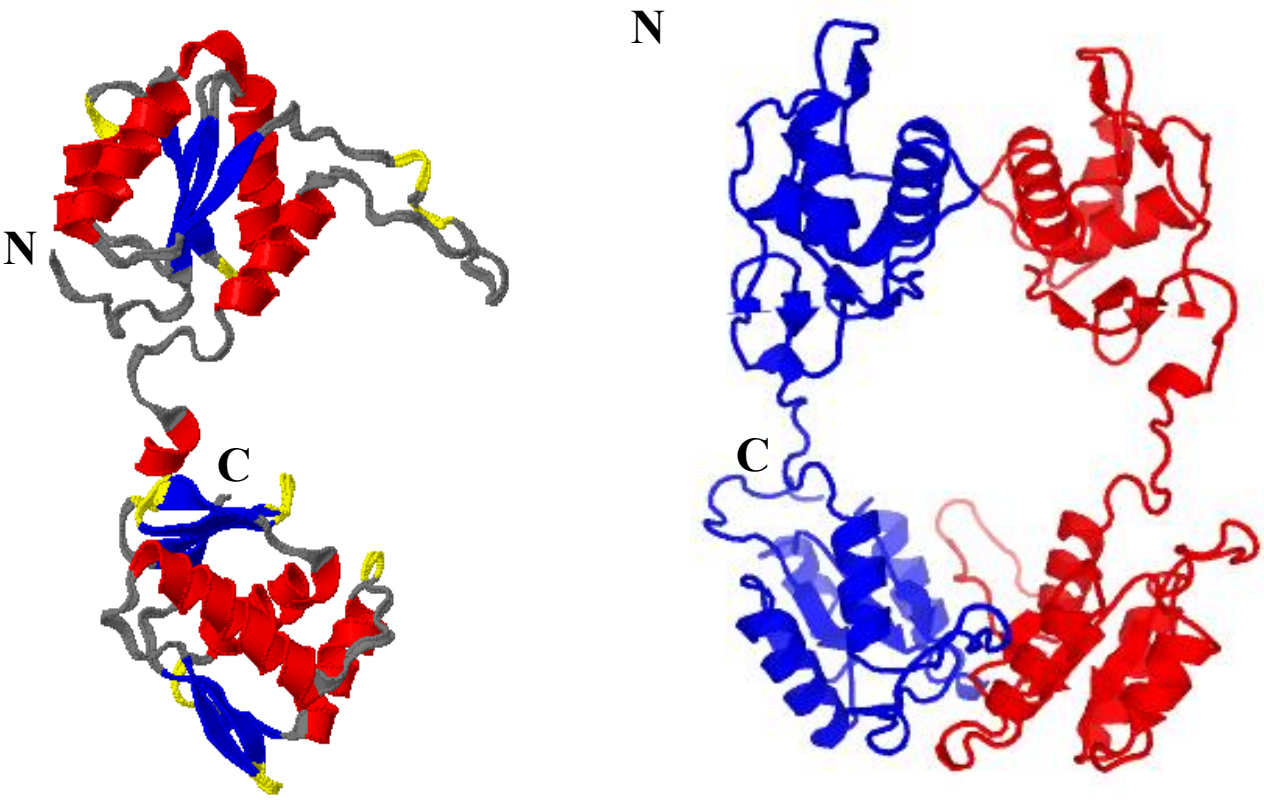


Fig. 3.11: Predicted *Salmonella* BaeR monomer and dimer structure

refinement based on loop modeling and overall structure refinement, Galaxy Homomer generates more detailed homo-oligomer models. Three-dimensional protein structure of a BaeR was also predicted using GalaxyWEB. Template-based modeling was done with refinements of loops and terminus region using *ab initio* modeling. The software yielded a dimer model which is similar to that of the crystal structure of *E. coli* BaeR as reported (21).

The predicted monomer and homo dimer model of *Salmonella* BaeR in In **Fig. 3.11** shows that *Salmonella* BaeR has an N-terminal domain that also contains a conserved Aspartate Residue as known from the sequence alignment data and a C-terminal domain that is used for DNA binding. The predicted dimer model is also shown (**Fig. 3.11**).

It can be seen that the predicted *Salmonella* BaeR is very similar to the *E. coli* BaeR (**Fig. 3.12**) due to the fact that both of them has receiver domains in N-terminal and a effector domain i.e., DNA binding domain in the C-terminal that are linked by a linker.

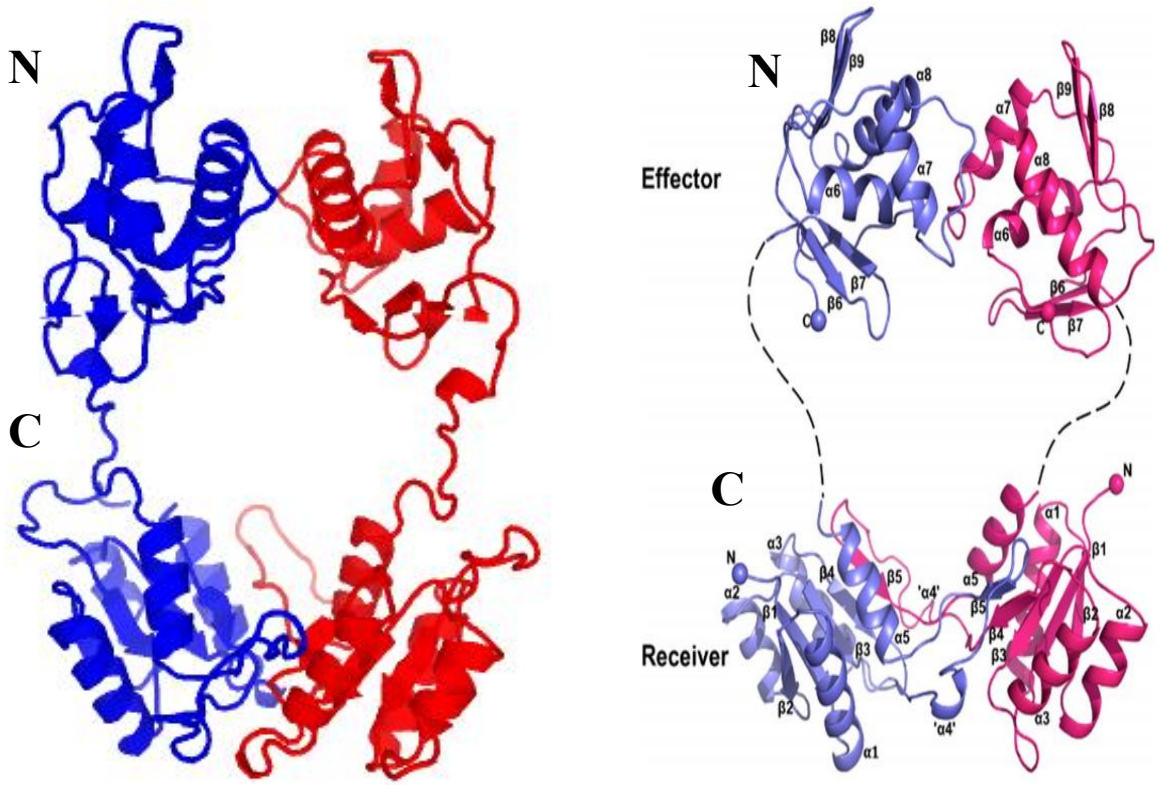


Fig. 3.12: Predicted *Salmonella* BaeR dimer structure and *E. coli* BaeR dimer. (21)

3.8 *Salmonella* BaeR forms dimers:

As one of the objectives is to characterize the *Salmonella* BaeR protein, it was discovered that *E. coli* BaeR forms a dimeric structure, while sequence alignment of *Salmonella* BaeR and *E. coli* BaeR shows 96.7 % similarity differing only in 8 amino acids. Thus we wanted to test whether *Salmonella* BaeR forms a dimeric structure or not.

A 12% semi-native gel was run where the same sample was loaded in two lanes, one with β-mercaptoethanol containing loading dye and another with loading dye without 2-mercaptoethanol to determine if any secondary interaction occurs among the BaeR proteins. In the SDS-Page gel image (**Fig. 3.13**) lane # 1 shows the protein ladder, the lane # 2 shows the BaeR protein with β-mercaptoethanol, and finally the lane #3 shows BaeR protein without β-mercaptoethanol where the sample was not heated before loading into the well. β-mercaptoethanol is a reducing agent that denatures disulfide bonds in proteins and heating denatures other interaction. In the lane # 3 where the sample was not heated and the reducing agent was not added, we observed a band near the 54 kDa region, indicating that *Salmonella* BaeR may form a dimeric structure as that of *E. coli* BaeR.

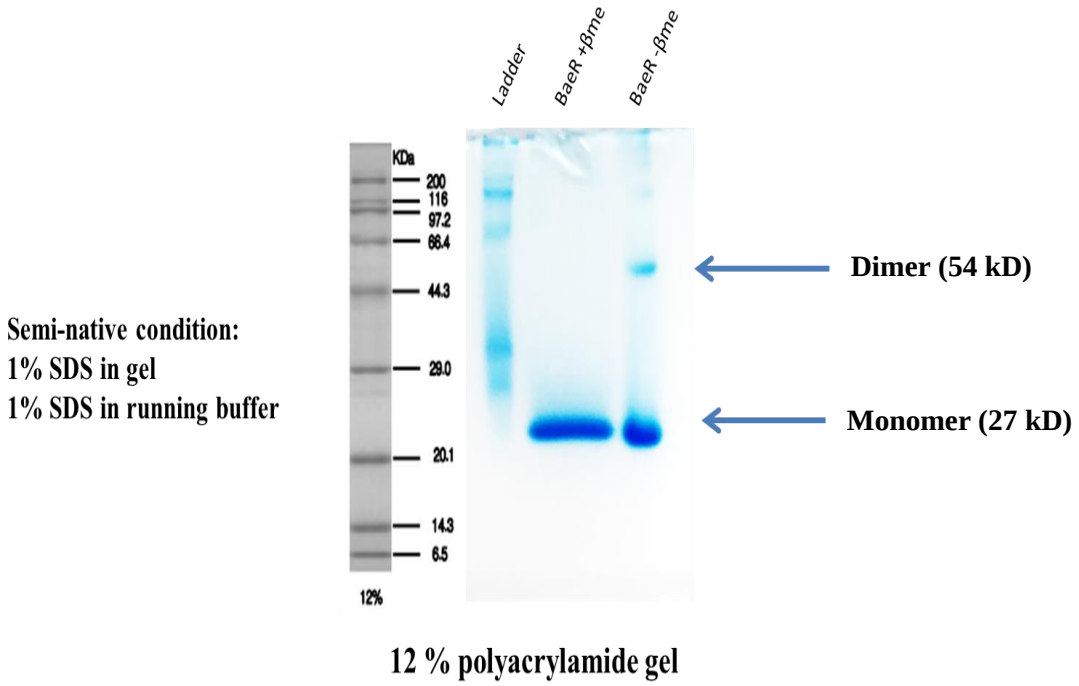


Fig. 3.13: Semi-native gel of *Salmonella* BaeR

3.9 *Salmonella* BaeR up-regulates AID in human (Raji) B-cells

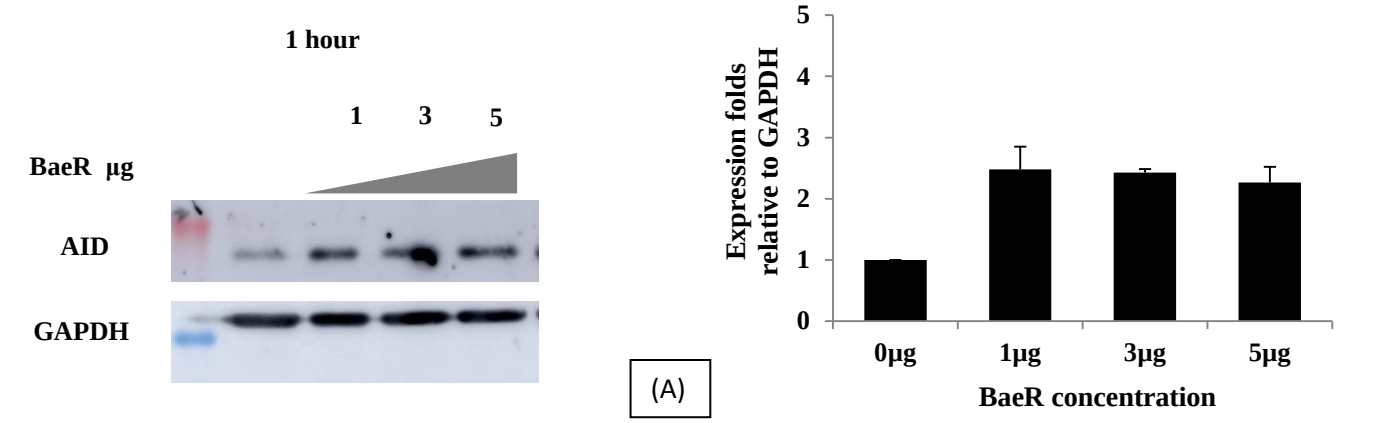
To check the effect of BaeR on aberrant AID expression present within Human B-cells (Raji cells). Human Raji B-cells were derived from the B-lymphocytes of a Nigerian patient suffering from Burkitt's lymphoma. Being a continuous immortalized cell line it is used as a model cell line for B-cell studies including B-cell lymphoma and expresses AID continuously. Thus, human Raji B-cells were chosen for our study. The Raji B-cells were

stimulated with different concentrations (1µg, 3 µg, 5 µg) of BaeR for different times (1 hour, 4 hours, 8 hours). Cells were collected, lysed using 1x RIPA buffer and protein samples were prepared, 80 µg of the samples were loaded and ran in a SDS gel, transferred in Nitrocellulose paper, probed with primary AID antibody and secondary Anti-AID antibody. After probing of AID with AID antibody, it was stripped and re-probed with GAPDH which acted as a loading control in order to know the relative change in the expression fold. Persistent upregulation of AID was observed in all hours in cells stimulated with 1 µg, 3 µg, 5 µg concentration of BaeR compared to that of control.

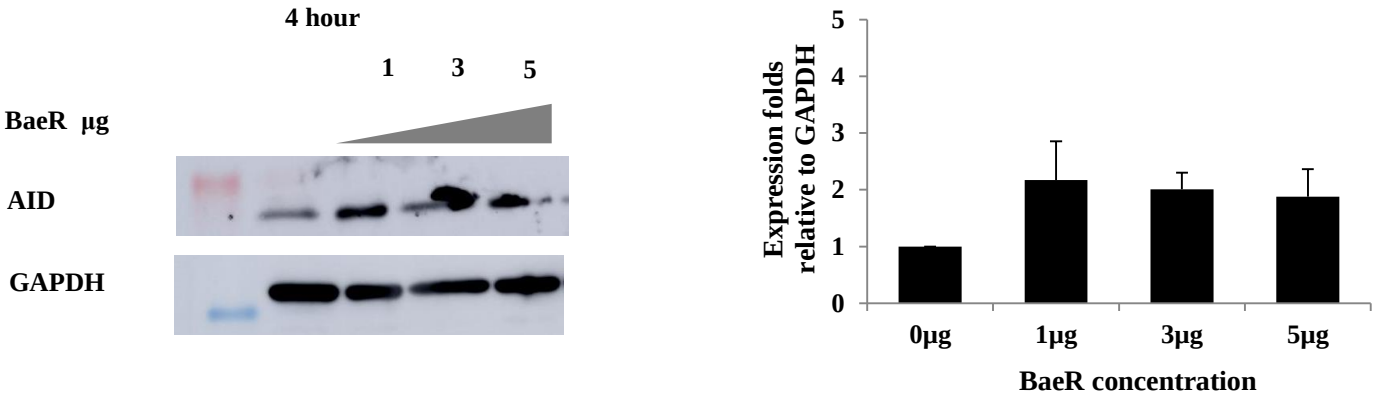
The immunoblots of AID and GAPDH of Human B-cells (**Fig. 3.14A**) are shown when they were treated with different concentration of BaeR (1µg, 3 µg, 5 µg) for 1 hour, followed by the graph (**Fig. 3.14 A**) showing upregulation of relative expression of AID with the change in BaeR concentration (1µg, 3 µg, 5 µg) when Raji B-cells were stimulated using BaeR for 1 hour. Relative expression levels increased 2.4 folds in case of cells treated with 1 µg, 3 µg, and 5 µg of BaeR for 1 hour with respected to untreated B-cells.

The immunoblots of AID and GAPDH of Human B-cells (**Fig. 3.14B**) are shown when they were treated with different concentration of BaeR (1µg, 3 µg, 5 µg) for 4 hours, followed by the graph (**Fig. 3.14 B**) showing upregulation of relative expression of AID with the change in BaeR concentration (1µg, 3 µg, 5 µg) when Raji B-cells were stimulated using BaeR for 4 hours. Relative expression levels increased 2.1 folds in case of cells treated with 1µg, 2 folds in case of cells treated with 3 µg of BaeR, 1.8 folds in case of cells treated with 5 µg of BaeR for 4 hours with respected to untreated B-cells.

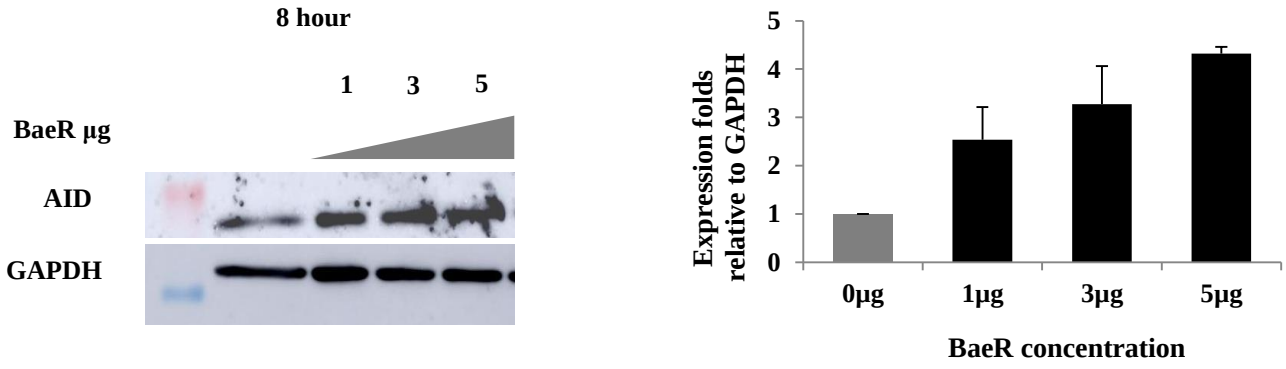
The immunoblots of AID and GAPDH of Human B-cells (**Fig. 3.14C**) are shown when they were treated with different concentration of BaeR (1µg, 3 µg, 5 µg) for 8 hours, followed by the graph (**Fig. 3.14 C**) showing upregulation of relative expression of AID with the change in BaeR concentration (1µg, 3 µg, 5 µg) when Raji B-cells were stimulated using BaeR for 8 hours. Relative expression levels increased 2.5 folds in case of cells treated with 1µg, 3.8 folds in case of cells treated with 3 µg of BaeR, 4.3 folds in case of cells treated with 5 µg of BaeR for 8 hours with respect to untreated B-cells.



(A)



(B)



(C)

Fig. 3.14: (A) Immunoblots of AID and GAPDH at 1 hour (Left), Expression folds of AID in BaeR stimulated B-cells (Right). (B) Immunoblots of AID and GAPDH at 4 hour (Left), Expression folds of AID in BaeR stimulated B-cells (Right). (C) Immunoblots of AID and GAPDH at 1 hour (Left), Expression folds of AID in BaeR stimulated B-cells (Right).

Chapter 4

Conclusion and Discussion

baeR gene from *Salmonella* was cloned into pET43 expression vector with a C-terminal 6X Histidine tag. The recombinant BaeR was expressed in *E. coli* Rosetta cells at 37 °C and purified using Ni-NTA columns. The recombinant BaeR formed a dimer as seen on a semi-native gel, which would be further confirmed by size exclusion chromatography.

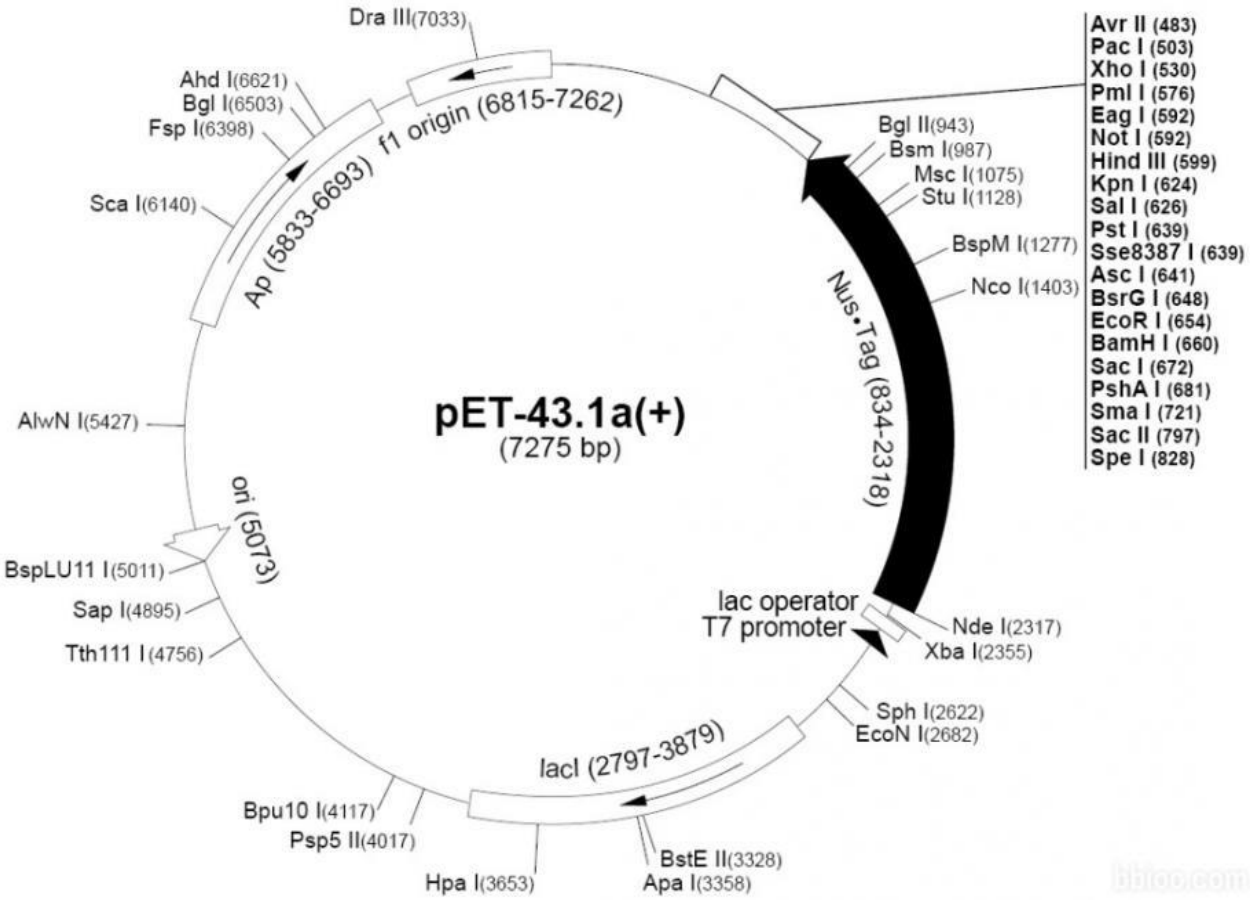
Salmonella infection is one of the most prominent foodborne infections. Very less is known about the role of B-cells in *Salmonella* infection. Reports have shown *Salmonella* infection activates B-cell. *Salmonella typhimurium* induces a profound antibody response against both protein and non-protein antigens. However, the response of B-cells and antibody production is less known in case of *Salmonella* infection. A number of *Salmonella* virulence factors have been reported to contribute in the *Salmonella* infection. *Salmonella* virulence factors can interact with BCR, independent of T-cell, and can elicit a profound B-cell response (19,20). Studies suggested that B-cells differentiated into short-lived plasma B-cells that contributed to bacterial elimination in retaliation to *Salmonella* infection in a T-cell independent manner. Accordingly, characterisation and elucidation of BaeR's functional role can give us an idea about the interaction of *Salmonella* with B-cells. Thus in this study, we aimed to give an insight into the structural characterisation of *Salmonella* BaeR and its functional role in B-cell modulation.

Structural characterisation of *Salmonella* BaeR revealed similarities with *E. coli* BaeR. Sequence alignment of *E. coli* BaeR with *Salmonella* BaeR resulted in 98.3 % similarity with the difference in only 8 amino acids. Secondary structure prediction also revealed close associations between the *E. coli* and *Salmonella* BaeR with the majority of the amino acids present within random coils and alpha-helix and only minor differences present between *E. coli* and *Salmonella* BaeR in amino acids present in the extended strand, beta turns and bend region. Reports of dimeric *E. coli* BaeR have already been published. With a similarity of > 90% we checked whether *Salmonella* BaeR is also present in a homo-dimer form. We ran a semi-native SDS PAGE and observed a band near the 54 kDa region, indicating that *Salmonella* BaeR may form a dimeric structure as that of *E. coli* BaeR.

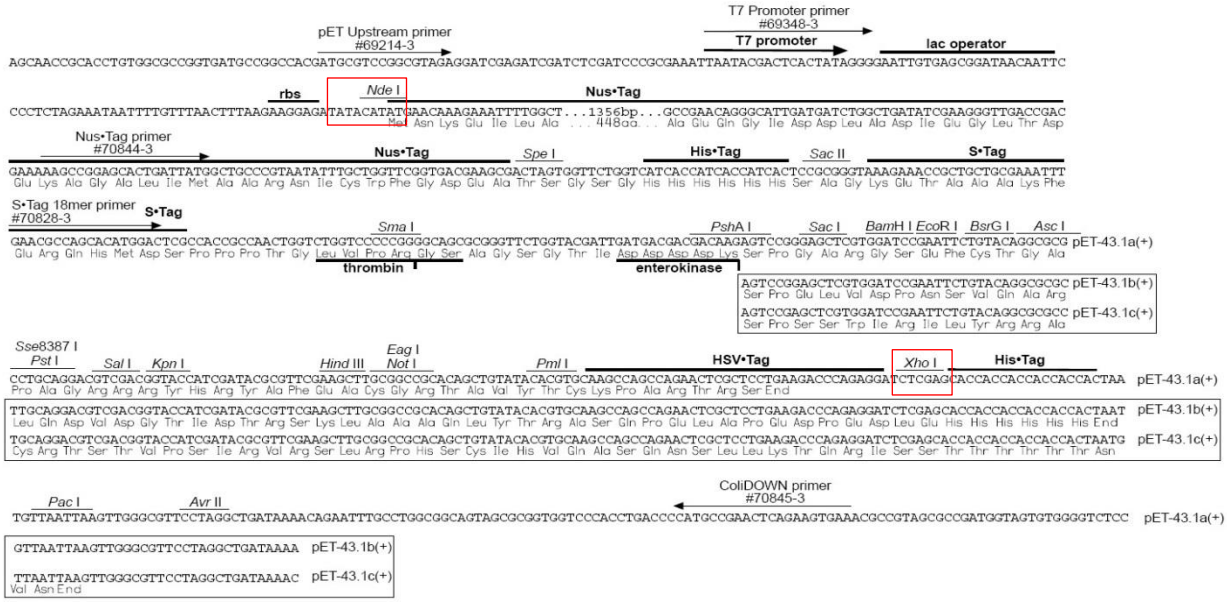
We also evaluated the effect of BaeR stimulation on AID expression via immunoblots. Endotoxin-free BaeR was used to stimulate the B-cells, due to the fact that LPS can interfere in the result as LPS has been reported to cause inflammation and antibody response. The results showed that AID was persistently up-regulated in B-cells at 1 hour, 4 hours and 8 hours when treated with BaeR of concentration 1 µg, 3 µg, and 5µg.

AID is the mutator enzyme that is mostly present in B-cells which causes deamination of cytosine to uracil. In order to correct the mutation, the error-prone repair machinery then act upon the uridine nucleotides to create single-stranded breaks at the switch regions, which are converted into double-stranded breaks upon proximity and are crucial intermediates during CSR. If the single-stranded breaks are far apart, they are repaired by base excision repair (BER) enzymes and mismatch repair (MMR) pathways. These mutation rates are high in immunoglobulin regions that give rise to SHM and CSR which is responsible for the production of specific high-affinity antibodies (17). It has already been reported that BaeR is a potent activator of NFκB, which is a transcription factor of AID (20). Aberrant expression of Activation-induced Cytidine Deaminase (AID) can be caused by several pathogenic factors like bacterial infections and proinflammatory chemicals. Aberrant AID expression can lead to various pathological conditions like hyper-IgM syndrome, systemic or organ-specific autoimmunity, allergies, asthma and cancer. In B-cells, aberrant AID expression can lead to mutations due to mistargeting of AID to certain oncogenes like *c-Myc*, *RhoH*, *Pim1*, and *Pax5* oncogenes that can cause B-cell lymphoma (23). Expression of AID is also associated with the transformation from the chronic stage of chronic myeloid leukemia (CML) to fatal B lymphoid blast crisis (LBC) (24). Stimulation of pro-inflammatory cytokines, which are upregulated by bacterial virulence factors, can induce aberrant expression of AID through the NFκB signaling pathway (24). It can be postulated that BaeR may activate NFκB in B-cells which may induce high AID expression that can lead to different pathological conditions including B-cell lymphomas. Further study can be done to elucidate the signaling pathway that is involved in the overexpression of AID in B-cells. Elucidation of the pathway would give us an idea about the effect of the overexpression of AID and the pathological condition associated with the aberrant AID expression.

Appendix



Vector map of pET43



pET43.1a expression/cloning region

TABLE

Table 1: List of primers

Serial No.	Primer Name	Sequence (5'-3')	Restriction site
1	PK663F	ggc**CATATG**actgaattacccattgatgaaaacac	*Nde*I
2	PK664R	atgc**CTCGAG**taccaggcgacacgcatc	*Xho*I

References:

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