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Optimization of Parameters for Accessory Cholera Enterotoxin (*Ace*) Protein Expression

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The accessory cholera enterotoxin (*Ace*) is one of the identified toxin of *Vibrio cholerae*. The gene coding for *Ace* toxin was successfully cloned in *Escherichia coli* expression system, pBAD/Thio-TOPO vector. The *Ace* toxin causes a mild cholera manifestation, increases the potential differences across the intestinal epithelium and alters ion transport. All these characteristics are proposed as contributing factors in causing diarrhea in human. In this study, the *Ace* protein was expressed in the *Escherichia coli* (*E. coli*) host strain, LMG194. Several parameters and conditions including host strain, temperature of culturing, arabinose concentrations, antibiotic concentrations and a suitable time course induction were performed in order to get the expression of the *Ace* protein. Secreted toxin obtained from the supernatant produced a fusion-protein (combination of the *Ace* protein and HP-thioredoxin protein) with a molecular mass of 34 kDa (the *Ace* protein is expected to have a molecular weight of 18 kDa). The fusion protein was detected by anti V5-HRP epitope tagged antibody. However, the *Ace* protein failed to be expressed in another *E. coli* host strain, TOP10 cell, which could be due to the deleterious effect of the toxin to the cell.

Key words: Cholera enterotoxin, accessory cholera enteroxin, pBAD vector

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Introduction

During the past decade, it has widely been accepted that the primary pathophysiological changes in cholera are caused by a soluble exotoxin elaborated by certain strains of *Vibrio cholerae*. This toxin is produced in the lumen of the small intestine of man and acts locally to produce a massive outpouring of small intestinal secretion resulting in net loss of body water and electrolytes leading to hypovolemic shock (Kadis *et al*., 1970). According to Koch *et al*. (1993) the major virulent factor produced by *Vibrio cholerae* is the cholera enterotoxin (CT), encoded by two contiguous genes forming the *ctxAB* operon. *V. cholerae* also produces a putative toxin known as zonula occludens toxin (*Zot*), which increases the permeability of small intestinal mucosa by affecting the structure of the intercellular tight junction (Baundry *et al*., 1992 and Fasano *et al*., 1991). Preliminary studies, using crude toxin extract in animal models indicated that *Ace* as the third toxin, acts by increasing transcellular ion transport (Trucksis *et al*., 1997). The genes encoding the toxins (*ace, zot* and *ctxAB*) and a core-encoded pilin (*cep*) and an *orfU* are located on a 4.5 kb "core region", flanked by one or more copies of a repetitive sequence called RSI (Trucksis *et al*., 1993). According to Trucksis *et al*. (1997) the *Ace* toxin can be expressed efficiently in the methylotrophic yeast *Pichia pastoris* but in the *Escherichia coli* expression system tested in the study, the *Ace* toxin obtained were unstable and resulted in cell lysis (Trucksis *et al*., 1993).

In this study, the *ace* gene was successfully cloned in pBAD/Thio-TOPO vector and expressed using *E. coli* host strain, LMG194. Thus, the objective of this study is to report the optimum conditions suitable for the expression of the Accessory cholera enterotoxin (*Ace*) in pBAD/TOPO Thiofusion *E. coli* expression system (Invitrogen, USA).

Materials and Methods

Bacterial isolates: Twenty *Vibrio cholerae* clinical isolates were obtained during an epidemic (1996-1998) in Malaysia from the patients with cholera. The isolates were collected from Hospital Kuala Lumpur and Institute of Medical Research (IMR), Kuala Lumpur, Malaysia. Eighteen of the isolates belongs to the Ogawa serotype, while two strains were Inaba and the O139 Bengal serotypes respectively.

Expression of fusion protein: Initially, the *ace* gene was cloned into *E. coli* expression vector, pBAD/Thio-TOPO and transformed into TOP10 *E. coli* host strain. Subsequently, the *Ace* clones were retransformed into different *E. coli* host strain called as LMG194. This was carried out in order to determine the host strain that is suitable for expression of *Ace* toxin. For both host strains, similar procedures were carried out to get the expression. Several modifications were made from the manufacturer's procedure (pBAD/TOPO® ThioFusion™ Expression System, Version B, Invitrogen, USA. Cat. No. K370-01), in order to get the expression. Firstly, the recombinants from TOP10 and LMG194 host strains containing the *Ace* clones were streaked on Luria Bertani (LB) and RM ([M9 Salts, 2% casamino acids, 0.2% glucose, 1mM $MgCl_2$], Invitrogen, USA) media respectively containing 100 $\mu g ml^{-1}$ ampicillin. Secondly, a single recombinant colony from both media was selected and inoculated in 2 ml of LB and RM broth containing 100μg ml^{-1} ampicillin and incubated overnight at 37°C with shaking (225-250 rpm) until the optical density at 600nm (OD_{600}) reached 1.5 OD_{600}. This was followed by inoculating each of the five flasks of 100 ml LB and RM broth (100 or 200μg ml^{-1} ampicillin) with 0.1 ml of overnight cultures of the recombinant colony. These flasks were incubated at 29 and 37°C with vigorous shaking until the turbidity was 0.5 OD_{600}. The recombinant cultures were then induced with different concentrations of arabinose (20, 2, 0.2, 0.02 and 0.002%) and were further incubated at 29 and 37°C. At various incubation periods (after 4, 24, 36, 48, 60, 66, 72, 84 and 90 hrs), aliquots of the cultures at 1 or 10 ml were spun at the maximum speed (13,000 rpm) for 30 seconds to collect the cells. The recombinant cultures were then stored at –20°C.

Protein analysis: The thawed recombinant cultures that were induced with different concentrations of arabinose were resuspended in 4 ml of lysis buffer [50 mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole] respectively, and sonicated until the cells were lysed to release the expressed protein from the cells. The lysis buffer was prepared fresh to lyse the cells. The sonicated cultures were centrifuged at a maximum speed of 13,000 rpm for 1 min at 4°C and the supernatant was transferred into a new tube. The pellet and supernatant were treated with 1X SDS and 2X SDS gel-loading buffer respectively, vortexed and then heated at 95°C for 5 min. The treated pellet and supernatant were then loaded on a 10% SDS polyacrylamide gel in a Laemmli buffer system (Laemmli, 1970) and electrophoresed at 100 Volt for 3 hours. The electrophoresed gel was then 'sandwiched' to the polyvinylidane difluoride (PVDF) membrane in a transfer buffer in order to facilitate the transfer of the protein from gel to the membrane. The procedure was according to a method that has been adapted from Towbin *et al*. (1979). The transferred protein which is a fusion protein was incubated with primary antibody anti V5-HRP (1 : 1250, Fermentas) and HRP conjugate anti-mouse (Amresco), as a secondary antibody. The detection was carried out using a Chromogenic Detection kit (Amresco).

Results

Expression of fusion protein: One of the six clones (TOP10/*ace*) identified by (Polymerase Chain Reaction) as containing *ace* gene tested for expression of *Ace* protein under different conditions that includes temperature of the cultures (29 and 37°C), antibiotic concentrations (100 to 200μg ml^{-1}), arabinose concentrations (0.02 to 20%) and time course induction (4 to 90 hours) was unable expressed.

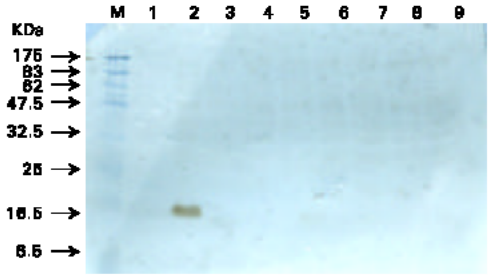

Fig. 1: Expression of *Ace* toxin in LMG194 *E. coli* host strain. Lane 1- Broad range protein marker (Fermentas), lane 2-positive control (0.02% arabinose, 0 hrs), lane 3- 16 kDa HP-thioredoxin protein when induced with 0.02% arabinose at 37°C for 4 hrs, lane 4- negative control, lane 5- sample (0.02% arabinose, 0 hrs), lane 6- sample (0.2% arabinose, 5 hrs), lane 7- sample (2.0% arabinose, 5 hrs), lane 8- sample (0.2% arabinose, 17 hrs), lane 9- sample (20% arabinose, 17 hrs), lane 10- sample (2.0% arabinose, 24 hrs).

On the other hand, the *Ace* fusion protein was successfully expressed in another *Escherichia coli* host strain, LMG194, which is named as LMG194/*ace* clone. In this study, the expression of the *Ace* protein was examined in a minimal medium, (which is called as RM medium) and found that the expression of arabinose-induced protein was produced in a stable pattern. The fusion protein was detected in the supernatant (Fig. 2) indicating that the protein is soluble. However, no protein was detected in the pellet. The band of fusion protein appeared at a size of approximately 34 kDa where the *Ace* protein's molecular weight is estimated to be 18,000 Dalton. The vector control is pBAD/Thio (4436 bp) which expressed a 16 kDa HP-thioredoxin protein when induced with 0.02% arabinose at 37°C and was detected in the pellet after 4 hours induction (Fig. 1, lane 3). The optimized conditions that enabled the expression of the fusion protein includes the temperature for culturing of construct LMG194/*ace*, which was at 29°C with a high antibiotic concentration of 200 μg ml^{-1}. In addition, the protein was expressed after 60 to 72 hours of

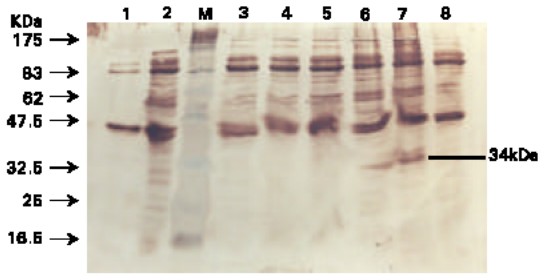

Fig. 2 : Expression of *Ace* toxin in LMG194 *E. coli* host strain. The fusion protein was detected in the supernatant indicating that the protein is soluble. However, no protein was detected in the pellet. Lane 1- sample (0.2% arabinose, 0hrs), lane 2- negative control (0.2% arabinose, 4hrs), lane 3- Broad range protein marker, lane 4- sample (0.2% arabinose, 4hrs), lane 5- sample (2.0% arabinose, 4hrs), lane 6- sample (0.2% arabinose, 24hrs), lane 7- sample (0.2% arabinose, 60hrs), lane 8- sample (0.2% arabinose, 72hrs), lane 9- sample (0.2% arabinose, 5hrs).

induction with 0.2% arabinose. The pBAD/Thio-TOPO vector encodes His-Patch thioredoxin as an N-terminal fusion partner. The thioredoxin fusion significantly increases the solubility of proteins that were difficult to express and improves the yield of protein production. The protein was detected from the recombinant culture at a volume of 10 ml but not from the recombinant culture at a volume of 1 ml as suggested by the protocol in the manual (pBAD/TOPO® ThioFusion™ Expression System, Version B, Invitrogen, USA. Cat. No. K370-01).

Discussion

A period of eight months was taken in optimizing the expression conditions for both *Escherichia coli* host strains, LMG194 and TOP10. The expression conditions tested were the induction time, induction temperature, concentration of arabinose and choice of host strain. To our knowledge this is the first report of expression of *Ace* protein produced in the pBAD/TOPO expression system. In this study, we have demonstrated the efficient expression of *Ace* toxin in pBAD/Thio-TOPO vector, *Escherichia coli* (*E. coli*) expression system and successfully expressed in LMG194 host strain. However, this toxin did not express into an *E. coli* host strain, TOP10. The different impacts of expression might be caused by the use of different host strains (Weickert *et al*., 1996). Some low copy number host strains generally produce lower protein expression level. The pBAD vector represent a simple and useful expression system for efficient repression, modulation and moderately high expression. The *E. coli* strain LMG194 allows the additional repression of low basal level expression of toxic genes (Guzman *et al*., 1995).

The pBAD vectors were constructed containing the P_{BAD} promoter of the arabinose operon and its regulatory gene, *ara*C (Schleif, 1992). In the presence of arabinose, transcription from the P_{BAD} promoter is turned on and in its absence transcription occurs at very low levels (Lee *et al*., 1991). The 34 kDa biotinylated *Ace* fusion protein was expressed after 60 to 72 hours of induction with 0.2% arabinose at 29°C compared to the expression of 16 kDa HP-thioredoxin protein which is 0.02% arabinose at 37°C after 4 hours induction. Previously, Trucksis *et al*. (1993) reported two forms of *Ace* toxin produced in the yeast expression system "*Pichia pastoris*" the predominant protein with a molecular weight of 18,000 Dalton and a second protein 9,000 Dalton. In another study, the expression of tetanus toxoid (TT) control of P_{BAD} was obtained at low temperature (20°C) after 3 hours of induction with 0.2% arabinose (Clark *et al*., 1997). However, the concentration of arabinose was considerably higher than the concentration used in the previous study (Johnson and Schlief, 1995). According to Guzman *et al*. (1995), several factors that contribute to an efficient repression of gene expression include the concentration of arabinose (the lower is better), the ability of the strain to degrade arabinose (*ara*$^+$ strains are better) and the physiological state of the culture due to the type of medium, amount and type of carbon sources present in medium. In this study, the *Ace* protein was detected after spun down from a large volume of induced recombinant cultures, 10ml instead of 1ml which is recommended by manufacturer's instruction. This is in accordance to Trucksis *et al*. (1997), whereby the *Ace* protein that was expressed in the methylotrophic yeast *Pichia Pastoris* produced only 7 mg of *Ace* toxin per liter. They also noted that the expression of *Ace* protein was highly dependent on the composition of the growth medium and the density of the culture at the time of induction of protein expression. Therefore, this investigation on the optimization of *Ace* toxin expression conclude that P_{BAD} promoter could induce high level of expression by using low level of arabinose.

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References

Baundry, B., A. Fasano, J. Ketley and J.B. Kaper, 1992. Cloning of a gene (*zot*) encoding a new toxic produced by *Vibrio cholerae*. Infection and Immunity, 60: 428-434.

Clark, M.A., F.R. Hammond, A. Papaioannou, N.J. Hawkins and R.L. Ward, 1997. Regulation and expression of human Fabs under the control of the *Escherichia coli* Arabinose Promoter, P_{BAD}. Immunotechnology, 3: 217-226.

Fasano, A., B. Baundry, D.W. Pumplin, S.S. Wasserman, B.D. Tall, J.M. Ketley and J.B. Kaper, 1991. *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. Proc. Natl. Acad Sci. USA., 88: 5242-5246.

Guzman. L.M., D. Belin, M.J. Carson and J. Beckwith, 1995. Tight regulation, modulation and high level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriology, 177: 4121-4130.

Johnson, C. and R.F. Schlief, 1995. In *vivo* induction kinetics of the arabinose promoter in *E. coli*. J. Bacteriology, 177: 3438-3442.

Kadis, V.W., W.E. Breitkreitz and O.J. Jonasson, 1970. Insecticide levels in human tissues of Alberta residents. Can. J. Public Health, 61: 413-416.

Koch, W.H., W.L. Payne, B.A. Wentz and T.A. Cebula, 1993. Rapid Polymerase chain reaction method for detection of *Vibrio cholerae* in Foods. Appl. and Environ. Microbiol., 59: 556-560.

Laemmli, U. K., 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Lee, K.Y., D. Hopkins, T.F. O'Brien and M. Syvanen, 1991. Gly-238-Ser Substitution Changes the Substrate Specificity of SHV Class A ß–lactamases. Prot. Struct. Func. Genet., 11: 45-51.

Schleif, R., 1992. DNA Looping. Annu. Rev. Biochem., 61: 199-203.

Towbin, H., T. Staehelin and Gordon, 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA., 76: 4350-4354.

Trucksis, M., T.L. Conn, A. Fasano and J.B. Kaper, 1997. Production of *Vibrio cholerae* Accessory cholera enterotoxin (*Ace*) in the Yeast *Pichia pastoris*. Infection and Immunity, 65: 4984-4988.

Trucksis, M., J.E. Galen, J. Michalski, A. Fasano and J.B. Kaper, 1993. Accessory cholera enterotoxin (*Ace*), the third toxin of *Vibrio cholerae* virulent cassette. Proc. Natl. Acad. Sci. USA, 90: 5267-5271.

Weickert, M.J., D.H. Doherty, E.A. Best and P.O. Olins, 1996. Optimization of heterologous protein production in *Escherichia coli*. Current Opinion in Biotechnology, 7: 494-499.

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