



ORIGINAL ARTICLE

Cloning and Expression of Recombinant Tick-Borne Encephalitis Virus-like Particles in *Pichia pastoris*

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Abstract

Objectives: The purpose of this study was to verify the feasibility of using the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter based *Pichia pastoris* expression system to produce tick-borne encephalitis virus (TBEV) virus-like particles (VLPs).

Methods: The complementary DNA encoding the TBEV prM signal peptide, prM, and E proteins of TBEV Korean strain (KrM 93) was cloned into the plasmid vector pGAPZαA, then integrated into the genome of *P. pastoris*, under the control of the GAP promoter. Expression of TBEV VLPs was determined by Western blotting using monoclonal antibody against TBEV envelope (E) protein.

Results: Recombinant TBEV VLPs consisting of prM and E protein were successfully expressed using the GAP promoter-based *P. pastoris* expression system. The results of Western blotting showed that the recombinant proteins were secreted into the culture supernatant from the *P. pastoris* and glycosylated.

Conclusion: This study suggests that recombinant TBEV VLPs from *P. pastoris* offer a promising approach to the production of VLPs for use as vaccines and diagnostic antigens.

1. Introduction

Tick-borne encephalitis virus (TBEV) belongs to the *Flavivirus* genus of the *Flaviviridae* family and can cause fatal encephalitis in humans in Europe, Russia, and Far East Asia [1,2]. In South Korea, TBEV was first isolated from wild rodents in 2006 [3]. The flavivirus genome contains a single, long, open reading frame that

encodes a polyprotein, which is cleaved into three structural proteins, i.e., the capsid (C), premembrane (prM) and envelope (E) proteins, and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [4]. In flaviviruses, the prM and E proteins play crucial roles in the assembly and secretion of the virions [5–8]. Several studies have demonstrated that flavivirus virus-like particles (VLPs), consisting of

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the prM and E proteins, have been shown to be similar to the native virions in the structural and functional features for infection [5,9–12]. Thus, flavivirus VLPs can be substituted for native virions in investigations into the biological features of flavivirus, such as vaccine study for the prevention of flavivirus-induced diseases.

Until recently, various expression systems, including mammalian cells and insect cells, have been used to produce TBEV VLPs as antigens [10,13,14].

Pichia pastoris is one of the most widely used systems for producing recombinant protein by heterologous expression [15]. This system offers several advantages in comparison with other eukaryotic expression systems, such as the production of large-scale target proteins in their native conformation and cost-efficiency, and the proven safety of yeast-expressed VLPs vaccines such as hepatitis B virus VLPs [16] and human papillomavirus VLPs [17]. For this reason, the *P. pastoris* expression system has been used for the production of flavivirus proteins including VLPs [18–21]. However, there are no reports on the production of TBEV VLPs from *P. pastoris*. In the current study, we investigated the expression of TBEV VLPs using the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter-based *P. pastoris* expression system.

To our knowledge, this is the first report on the successful expression of TBEV VLPs in *P. pastoris*.

2. Materials and methods

2.1. Viruses and cells

The TBEV Korean isolate, KrM 93 strain (GenBank accession No. HM535611), was propagated in the brains of suckling mice and BHK-21 cell as described previously [22]. The infected cell culture medium was used for RNA extraction.

2.2. Yeast strain and plasmid vector

The *P. pastoris* host strain X33 (Invitrogen, Carlsbad, CA, USA) was used as the expression host in this study. The expression vector pGAPZαA (Invitrogen) contains the selectable marker Zeocin (Invitrogen), which is bifunctional in both *P. pastoris* and *Escherichia coli*, the GAP promoter, and the alcohol oxidase I (*AOX1*) transcription termination regions. *E. coli* transformants were selected on low salt Luria-Bertani agar plates containing 25 µg/mL Zeocin. *P. pastoris* transformants were selected on YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1M sorbitol, 2% agar, and 100 µg/mL Zeocin) and *P. pastoris* liquid cell cultures were grown in YPD broth (1% yeast extract, 2% peptone, and 2% dextrose) with Zeocin.

2.3. Construction of recombinant expression vector

The coding sequence for the signal peptide of prM, prM, and E proteins was amplified from KrM 93 strain viral RNA by reverse transcription-polymerase chain

reaction (RT-PCR) using SuperScript III First-strand synthesis system for RT-PCR (Invitrogen) and *Ex Taq* DNA polymerase (Takara, Shiga, Japan) according to the manufacturer's instructions, and the following primers: KrM93SS-F 5'-GAC **TTC GAA ATG** GTT GGC TTG CAA AAA-3' (*Bst* BI site in bold and start codon in italics) and KrM93E-R 5'-GAA **TCT AGA** GCT GCC CCC ACT CCA AGG-3' (*Xba* I site in bold). The PCR product (named as 93prM-E) was first cloned into pCR 2.1-TOPO plasmid (Invitrogen) and then subcloned into pGAPZαA following enzymatic digestion using *Bst* BI and *Xba* I (New England BioLabs Inc., Beverly, MA, USA) to construct the full-length 93prM-E clone downstream of GAP promoter, which was designated as pGAPZαA/93prM-E (Figure 1A). The plasmid inserts were confirmed by DNA sequencing.

2.4. Yeast transformation

The plasmid pGAPZαA/93prM-E was linearized with *Bgl* II (New England BioLabs) and transformed into *P. pastoris* X33 using *Pichia* EasyComp Kit (Invitrogen) according to the manufacturer's instructions. The transformed yeast cells were incubated in YPDS agar containing 100 µg/mL Zeocin at 30°C for 3–4 days. Zeocin-resistant yeast colonies were selected and identification of the insert in these Zeocin-resistant transformants was checked by colony PCR.

2.5. Expression of recombinant TBEV E protein in *P. pastoris*

Positive transformants, selected as described previously, were inoculated in 5 mL YPD broth with 100 µg/mL Zeocin with shaking (250 rpm) at 30°C overnight. These cultures were transferred to 500 mL of YPD broth with Zeocin with shaking (250 rpm) at 30°C for 48 hours. The culture supernatant and cell pellet were collected by centrifugation at 10,000 × *g* for 10 minutes. The cell pellet was disrupted using Yeast PE LB (G-Biosciences, St. Louis, MO, USA) according to the manufacturer's instructions. The lysate was clarified by centrifugation at 20,000 × *g* for 30 minutes at 4°C. The culture supernatant was precipitated by using Amicon Ultra-15 Centrifugal Filter Units with 30 kDa membrane (Millipore, Billerica, MA, USA) according to the manufacturer's instructions.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis

The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a reduced 4–12% Bis-Tris Gel (Invitrogen). Separated protein bands were transferred onto a 0.45-µm polyvinylidene difluoride membrane using the iBlot system (Invitrogen) according to the manufacturer's instructions. The membrane was blocked with 5% skim milk in Tris buffered saline with 0.05%

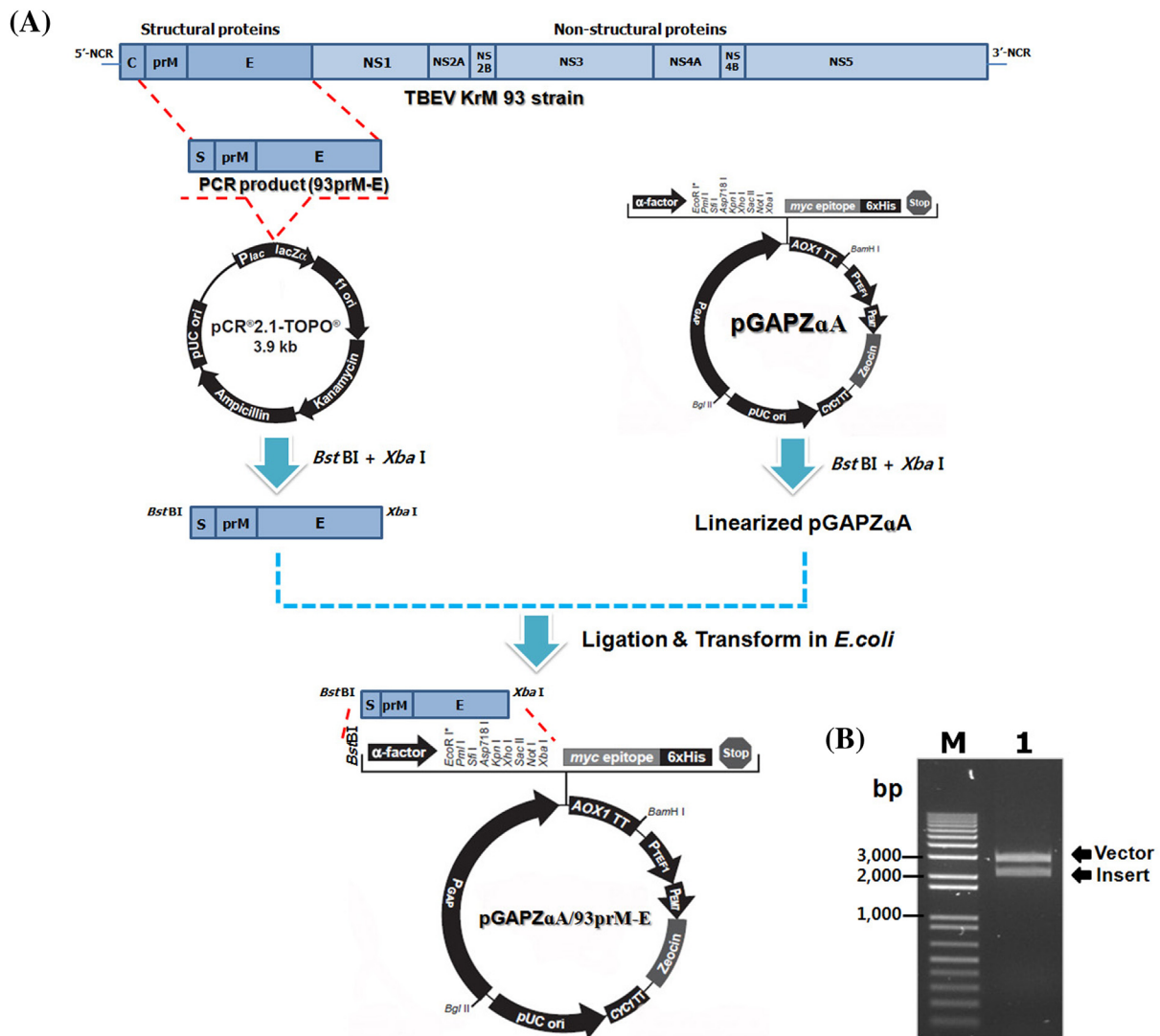


Figure 1. Construction of recombinant plasmid pGAPZ α A/93prM-E. (A) Scheme for cloning the 93prM-E fragment into the pGAPZ α A vector. (B) Confirmation of vector and insert by digestion with the restriction enzymes, *Bst* BI and *Xba* I. bp = base pairs, E = envelope protein; Lane M = 1 Kb DNA plus ladder; Lane 1 = pGAPZ α A/93prM-E digested with *Bst* BI and *Xba* I; prM = premembrane protein; S = the signal peptide of prM.

Tween-20. TBEV E protein was detected using monoclonal anti-TBEV E antibody (8A7 mAb, in-house) as the primary antibody and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Promega, Madison, WI, USA) as the secondary antibody. The bands were visualized by enhanced chemiluminescence (GenDEPOT, Barker, TX, USA).

2.7. Analysis of glycosylation patterns of recombinant TBEV E protein

Glycosylation patterns of recombinant E protein in lysate and supernatant were analyzed by digestion using *N*-glycosidase F (PNGase F) and endoglycosidase H (Endo H; New England BioLabs) according to the manufacturer's instructions. Glycosidase-treated protein samples were analyzed by SDS-PAGE and Western blotting as described previously.

3. Results

3.1. Construction of recombinant plasmid containing TBEV *prM* and *E* genes

The full-length *prM* and *E* genes of TBEV KrM 93 strain about 2 kb in size were amplified by using the RT-PCR method previously described. The 93prM-E fragment was then inserted into the yeast expression vector pGAPZ α A to construct a recombinant plasmid pGAPZ α A/93prM-E. The insert was confirmed by digestion with the restriction enzymes, *Bst* BI and *Xba* I (Figure 1B).

3.2. Expression of recombinant TBEV VLPS in *P. pastoris*

The pGAPZ α A/93prM-E plasmid was transformed into *P. pastoris* and positive clones were selected. Yeast

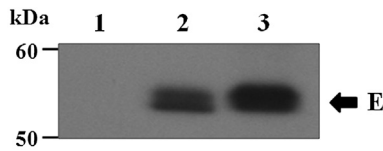


Figure 2. Western blot analysis on the expression of tick-borne encephalitis virus E protein in *Pichia pastoris*. Lane 1 = plasmid alone control; Lane 2 = sample from the cell lysate; Lane 3 = sample from the culture supernatant.

cell lysate and culture supernatant were collected and analyzed for expression of E proteins by Western blotting using TBEV mAb 8A7. As shown in Figure 2, the specific recombinant E protein bands with a molecular mass of ~ 55 kDa were detected in both the lysate and supernatant, but not in the plasmid-alone control. This result demonstrated that the recombinant TBEV VLPs were secreted into the culture supernatant from the yeast cell.

The glycosylation state of E protein in TBEV VLPs were examined with Endo H, which is specific for high mannose-type oligosaccharides and PNGase F, which cleaves both high mannose- and complex-type oligosaccharides. Comparison of glycosidase-treated recombinant TBEV E protein from yeast cell lysate and culture supernatant by SDS-PAGE and Western blotting indicated that the recombinant E protein was glycosylated in the *P. pastoris* expression system (Figure 3).

4. Discussion

VLPs have been developed as effective vaccine candidates, because they mimic the organization and conformation of native virion without containing the viral full genome, and thus are a safe and highly immunogenic antigen. Among the various protein expression systems for VLPs production, the yeast expression system as an alternative eukaryotic expression system is a well-established platform for the efficient production of heterologous viral glycoproteins. Previous studies have reported that this system has been used for the generation of flaviviral glycoproteins [18–21]. Among the yeast expression systems, the protein expression level in the *P. pastoris* expression

system was reportedly higher than that in another yeast *Saccharomyces cerevisiae* [23] and the GAP promoter based *P. pastoris* expression system can improve the protein yield compared to the *P. pastoris* by using methanol-inducible *AOXI* promoter [24]. We therefore have selected the *P. pastoris* expression system under the control of the GAP promoter to produce the TBEV VLPs in this study.

Here, we demonstrated the efficient production of TBEV VLPs, consisting of the prM and E proteins by using the GAP promoter-based *P. pastoris* expression system. The KrM 93 strain, which belongs to the Western subtype of TBEV isolated in South Korea [22], was used for production of recombinant proteins. As a main structural protein of flavivirus, the E protein plays important roles in receptor binding, membrane fusion activity, and immunogenicity [25,26]. The *prM* gene was included in the recombinant plasmid pGAPZ α A/93prM-E because E protein requires coexpression of the prM protein to maintain its native conformation and the protection of E protein from conformational alteration during transportation through the acidic compartments of the trans-Golgi network [27–29].

The results of Western blotting indicated that the TBEV VLPs were secreted into the culture supernatant from *P. pastoris*, suggesting that the signal peptide of prM can induce the secretion of TBEV VLPs, and coexpression of prM and E proteins in yeast cells may play an important role in virus particle release as shown in previous studies [5–9]. In addition, analysis of glycosylation of TBEV E protein in *P. pastoris* transformed with plasmid pGAPZ α A/93prM-E showed that the recombinant E protein from *P. pastoris* was glycosylated by eukaryotic posttranslational modifications (Figure 3).

This is the first study to report the successful cloning and expression of TBEV VLPs by using the GAP promoter-based *P. pastoris* expression system, and this study demonstrated that recombinant TBEV VLPs from *P. pastoris* offer a promising approach to the production of VLPs for use as vaccines and diagnostic antigens. Further study will be required for the purification of TBEV VLPs from *P. pastoris* and to identify the immunogenicity in a mouse model.

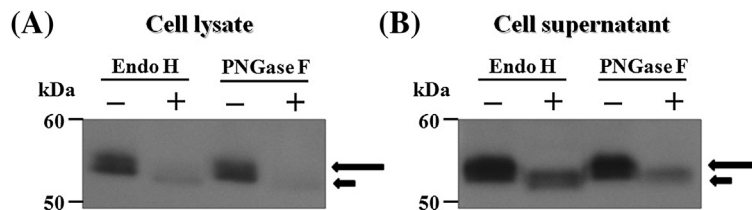


Figure 3. Analysis of glycosylation of tick-borne encephalitis virus E proteins in *Pichia pastoris* transformed with plasmid pGAPZ α A/93prM-E. Samples from (A) the cell lysate and (B) the cell supernatant were treated with Endo H (+) or PNGase F (+) and compared with untreated controls (-) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. \blackleftarrow = E protein; \blackleftarrow = deglycosylated E protein; Endo H = endoglycosidase H; PNGase = N-glycosidase F.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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