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An optimized expression vector for improving the yield of dengue virus-like particles from transfected insect cells

Nicha Charoensri[a], Amporn Suphatrakul[b], Rungtawan Sriburi[c], Thippawan Yasanga[d], Jiraphan Junjhon[e], Poonsook Keelapang[c], Utaiwan Utaipat[f], Chunya Puttikhunt[b,g], Watchara Kasinrerk[h,i], Prida Malasit[b,g], Nopporn Sittisombut[b,c,*]

[a] Center for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand
[b] Medical Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Bangkok 10700, Thailand
[c] Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
[d] Medical Science Research Equipment Center, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand
[e] Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok 10400, Thailand
[f] Research Institute for Health Sciences, Chiang Mai University, Chiang Mai 50200, Thailand
[g] Dengue Hemorrhagic Fever Research Unit, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
[h] Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand
[i] Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency at the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand

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ABSTRACT

Recombinant virus-like particles (rVLPs) of flaviviruses are non-infectious particles released from cells expressing the envelope glycoproteins prM and E. Dengue virus rVLPs are recognized as a potential vaccine candidate, but large scale production of these particles is hindered by low yields and the occurrence of cytopathic effects. In an approach to improve the yield of rVLPs from transfected insect cells, several components of a dengue serotype 2 virus prM + E expression cassette were modified and the effect of these modifications was assessed during transient expression. Enhancement of extracellular rVLP levels by simultaneous substitutions of the prM signal peptide and the stem-anchor region of E with homologous cellular and viral counterparts, respectively, was further augmented by codon optimization. Extensive formation of multinucleated cells following transfection with the codon-optimized expression cassette was abrogated by introducing an E fusion loop mutation. This mutation also helped restore the extracellular E levels affected negatively by alteration of a charged residue at the pr-M junction, which was intended to promote maturation of rVLPs during export. Optimized expression cassettes generated in this multiple add-on modification approach should be useful in the generation of stably expressing clones and production of dengue virus rVLPs for immunogenicity studies.

1. Introduction

Dengue virus (DENV), a member of the genus *Flavivirus*, is an important mosquito-borne human pathogen. Dengue is endemic and seasonally epidemic in many highly populated regions. With climate changes and continuing expansion in global travel, the number of people at risk is expected to increase and the burden from dengue intensifies. Vaccine for the prevention of dengue has been under development for decades, but a licensed product is not yet available.

A number of dengue vaccine candidates are being tested at various stages of pre- and clinical trials (Schmitz et al., 2011). Sanofi Pasteur's tetravalent Yellow fever–dengue chimeric vaccine candidates have reached phase III clinical trials (Guy et al., 2011). Other candidates, which are aimed at avoiding the untoward effects of live vaccines, include truncated E protein, recombinant domain III

* Corresponding author at: Department of Microbiology, Faculty of Medicine, Chiang Mai University, 110 Intawaroros Street, Chiang Mai 50200, Thailand.
Tel.: +6653945334; fax: +6653217144.
E-mail address: nsittiso@mail.med.cmu.ac.th (N. Sittisombut).

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0166-0934/

Table 1
Primers used in this study.

Primer	Sequence (5′-3′)
5′ SP_{DENV}	GGCGGTACCCCAACCATGAGATCTGCAGGCATGATCATTATGCTGATTCCAACAGTGATGGCGTTCCATTTAACCACACGT
5′ SP_{KRV}	GGCGGTACCCCAACCATGCGTAGTGGACCGAACCTTGTCATGATCATTGGGCTCTTGGCTCTTGGCTTATGCTATGGTTTCCATTTAACCACACGT
5′ SP_{mel}	GGCGGTACCCCAACCATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTTGTATACATTTCTTTCATCTATGCGTTCCATTTAACCACACGT
5′ SP_{DefA}	GGCGGTACCCCAACCATGAAGTCGATCACTGTCATTTGTTTCCTGGCTCTGTGCACCGTGGCCATCACTGCCTTCCATTTAACCACACGT
3′ Env	GGCGAGCTCTTAGGCCTGCACCATGACTCC
3′ Env80	CGGGAGCTCTTAGGTCGACCCTTTCTTAAACCAGTTGAGC
5′ SalI-JE20	CGGGTCGACCCTGGGCAAGGCCTTTTC
3′ XbaI-JE20	GGCTCTAGATTAAGCATGCACATTGGTCGC
3′ EcoRV-JE20	GCGGATATCTTAAGCATGCACATTGGTCGC
OptD2-H387L-F	TGCGGCCTGTTCGGCAAGGGTGGTATCGTGACC
OptD2-H387L-R	CCCTTGCCGAACAGGCCGCAACCGTTACCCCAA
OptD2-A203E-F	CGTCGTGAAAAGCGTTCGGTGGC
OptD2-A203E-R	ACGCTTTTCACGACGGTGTTCACC

of the E protein, DNA vaccines and recombinant virus-like particles (rVLPs). Among these, particulate vaccine candidates may induce a more efficient neutralizing antibody response than subunit preparations (Zlatkovic. et al., 2011).

DENV rVLPs are generated by expressing the prM + E coding region in mammalian cells (Konishi and Fujii, 2002; Purdy and Chang, 2005; Wang et al., 2009; Zhang et al., 2011), insect cells (Kuwahara and Konishi, 2010) or yeast cells (Liu et al., 2010). Large-scale production is hampered by low yield and cytopathic effects in (prM + E)-expressing cells (Konishi et al., 2001). Several approaches have been employed for improving the yield of rVLPs, including replacements of the prM signal peptide or the stem-anchor region of E with Japanese encephalitis virus (JEV) counterparts (Chang et al., 2003; Zhang et al., 2011), alteration of the furin consensus sequence at the pr-M junction (Konishi and Fujii, 2002), and mutation of the E fusion loop (Trainor et al., 2007; Chiou et al., 2008).

Previous attempts to increase the yield of DENV rVLPs by individual component modifications of the prM + E gene did not achieve the high levels required for large-scale production. In this multiple add-on modification approach, we assessed whether concurrent modifications of the prM signal peptide and E stem-anchor could lead to enhanced rVLP production, and whether additional increases would be possible with codon optimization. We then determined if extensive fusion of transfected cells, an expected consequence of codon optimization, could be reduced by a fusion loop mutation. Finally, the role of a prM cleavage-enhancing mutation (Junjhon et al., 2008) in counteracting a tendency toward poor cleavage of prM during expression of the codon-optimized prM + E gene was examined.

2. Materials and methods

2.1. Construction of (prM + E)-expressing plasmids

A (prM + E)-expressing plasmid was generated by introducing an expression cassette, which contained an insect ribosomal binding site, the prM signal sequence and the prM + E gene from DENV-2 strain 16681 (Sriburi et al., 2001) or a recent isolate (strain 03-0420), into pIE1-SV (Huynh and Zieler, 1999) (Fig. 1). In single chimeric plasmids, the prM signal sequence was replaced with those of the Kamiti river virus (KRV) prM, mellitin or defensin A by employing corresponding forward primers in the amplification reactions (Tables 1 and 2). In dual chimeric plasmids, the stem-anchor region of E was derived from JEV strain SA14-14-2 (Table 1).

A codon-optimized expression cassette was designed based on *Aedes albopictus*-preferred codons (Nakamura et al., 2000), consisting of the defensin A signal sequence, prM- and EDI-III-coding regions of DENV-2, and a JEV-derived stem-anchor region. This cassette was introduced into pIE1-SV-blasticidin, which was constructed by introducing a blasticidin resistance gene from pIB/V5-His (Invitrogen, Carlsbad, CA, USA) into pIE1-SV.

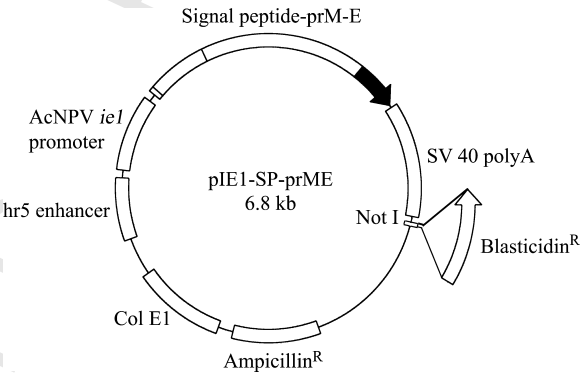

Fig. 1. Schematic representation of the (prM + E)-expressing plasmid. An expression cassette containing the prM signal sequence from the C-terminus of the capsid protein, and the (prM + E)-coding region of DENV-2 was inserted into pIE1-SV to generate pIE1-SP-prME. The blasticidin resistant gene was introduced at the NotI site in pIE1-SP-prME-blasticidin. The shaded area represents the 99-residue stem-anchor region of E that was substituted with the corresponding segment from JEV in some constructs.

2.2. Cells and transfection

C6/36 cell line (Igarashi, 1978), was maintained at 29 °C in Leibowitz's L-15 medium supplemented with tryptose phosphate broth and 10% fetal bovine serum (FBS). Sf9 cells (Novagen, Darmstadt, Germany) were maintained in an insect cell medium (EMD Chemicals, San Diego, CA, USA) supplemented with 10% FBS. Co-transfection of a (prM + E)-expressing plasmid and a luciferase-expressing plasmid into C6/36 cells was performed by employing Lipofectamine 2000 (Invitrogen). Sf9 cells were transfected using FuGene HD (Roche, Indianapolis, IN, USA). A blasticidin-resistant C6/36 clone expressing stably the dual chimeric, codon-optimized prM + E cassette was established to aid in the assessment of the particulate nature of secreted viral envelope proteins. Transfected C6/36 cells were seeded in L-15 medium supplemented with 10% tryptose phosphate broth, 1.5% FBS, and antibiotic solutions in the presence of 25 μg/ml of blasticidin (Invitrogen). Resistant colonies

Table 2
Origins and amino acid sequences of signal peptides employed in this study.

Protein	Source	Sequence	Length (residues)
prM	Dengue virus	MRSAGMIIMLIPTVMA	16
prM	Kamiti river virus	MRSGPNLVMIIGLLALGLCYG	21
Mellitin	*Apis cerana cerana*	MKFLVNVALVFMVVYISFIYA	21
Defensin A	*Aedes aegypti*	MKSITVICFLALCTVAITA	19