ISOLATION AND CHARACTERIZATION OF ANTI-DENGUE VIRUS PROTEINS FROM *CHROMOBACTERIUM PANAMA*

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ABSTRACT

Dengue virus (DENV) is the most important viral pathogen transmitted by *Aedes sp.* mosquitoes and the causative agent of dengue fever. According to WHO, over 40% of the world population is at risk from dengue and about 2.5% of those affected die each year. Our laboratory has previously identified a novel bacterium in the gut of field caught *Aedes* mosquitoes that had interesting properties with regards to vector competence. It was identified as *Chromobacterium Panama* (*Csp_P*), and studies have shown that it inhibit DENV infection in the mosquito. Further studies showed that the *Csp_P* culture supernatant could inhibit DENV infection *in vitro*. Based on these findings, we characterized several biochemical features and the mode of action of the putative anti-DENV factors. The *Csp_P*-derived anti-DENV factors were heat-stable at 70°C and the activity was enriched in the protein fraction, with molecular weights ranging from 50-100kDa. The activity seems to be associated with a *Csp_P* secreted multiprotein complex or protein oligomer. We also documented an apparent loss of the DENV envelope protein upon exposure to this protein fraction, which most likely account for abolished viral attachment to host cells. Additionally, proteomic analysis of the fraction identified 33 unique *Csp_P* proteins, including an extracellular cholesterol oxidase that has been indirectly linked to viral replication in several studies. Several bacterial metalloproteases were also identified, and may be responsible for the proteolytic degradation of the DENV envelope protein. Together, our study characterized an important microbial-derived protein extract with anti-DENV activity *in vitro*.

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INTRODUCTION

Dengue virus (DENV) is a mosquito-borne, positive-sensed RNA virus belongs to the *Flaviviridae* family. The family also include the yellow fever, West Nile, Japanese encephalitis, tick born encephalitis, and Zika viruses (Blitvich & Firth, 2015). Infection with DENV often causes dengue fever (DF), which is characterized by biphasic fever, headache, pain in various parts of the body, rash, lymphadenophathy, and leukopenia (Lei et al., 2001). Therefore, the name of dengue was initially "dandy fever" based on symptoms of infected individuals during the West Indian epidemic of 1827 (Smart, 1877). In most cases, DENV is self-limited. However, there is a risk of progressive development (2-2.5% of the cases) into severe manifestations such as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (Lei et al., 2001). The disease is transmitted among humans via competent female *Aedes* mosquitoes, with *Ae. aegypti* being the primary vector, a cosmotropical mosquito that thrives in urban environments(Lam, 2013) (Bowman, Donegan, & McCall, 2016). According to the World Health Organization (WHO), approximately half of the world's population is at risk of infection with DENV, along with 22,000 deaths occur each year mainly among children. The incidence of dengue has increased by 30-fold over the last 50 years, and this is attributed largely to urbanization, global warming and international travels (Abd Kadir, Yaakob, & Mohamed Zulkifli, 2013). In general, dengue is prevalent in tropical and subtropical regions, disseminating in more than 100 endemic countries in the southeast Asia, the western Pacific, the Americas, the Middle East and Africa (Guzman & Harris, 2015) (Figure 1). Currently, no therapeutics for dengue infection are available,

while clinical management remains supportive care (Anders & Hay, 2012). Nevertheless, nearly a half of patients with dengue have limited access to standard treatment (Lam, 2013). Until the recent advance in dengue vaccine development, dengue control has been solely relied on the control of its *Aedes* vectors, implying an unmet need for therapeutic interventions (Nedjadi et al., 2015). My study focuses on a novel *Chromobacterium. sp*, bacterial strain (*Csp_P*) which produces metabolite(s) that reduce DENV replication in both mosquito and cell cultures (Ramirez et al., 2014).

The global burden of dengue

To date, it has been estimated that nearly 4 billion people worldwide are at risk of dengue infection, yet the incidence of dengue is underestimated and is continuously increasing (Brady et al., 2012). Increased global incidence of dengue is due to a number of factors, including the uncontrolled vector (the expanding range and population of the *Aedes* mosquito along with increased temperature and precipitation) and the amplified transmission dynamic (rapid population growth as a result of rural to urban migration, leading to uncontrolled urbanization, increasing international travel due to globalization, enabling infected human hosts to move the viruses long distances more) (Lam, 2013). Unlike malaria, which is more prevalent in rural areas, cases of dengue are more common in urban and sub-urban areas. It is much more complicated to control dengue in highly populated areas in cities (Abd Kadir et al., 2013).

Challenges to effectively control dengue also attributed by the heterogeneous dengue serotype circulating. There are 4 antigenically and phylogenetically distinct but close-related serotypes, DENV1-DENV4 (Satterfield, Dawes, & Milligan, 2016). Infection with one serotype confers long-term protective immunity against infecting serotype, and temporary immunity to the three other serotypes (Fibriansah et al., 2014) (Guzman & Harris, 2015). The postulated mechanism for secondary infections associated with severe disease has been established as antibody-dependent enhancement (ADE) (Diamond & Pierson, 2015). During a primary dengue infection, cross-reactive, poorly-neutralizing antibodies are generated. During the secondary infection with a different DENV serotype, the Fc portion of the cross-reactive antibodies can bind to virions and facilitate virus entry into FcR-bearing target cells (Botting & Kuhn, 2012) (Lau et al., 2015). Therefore, incomplete infection-induced immunity failed to cover all four serotype theoretically could predispose an individual to severe disease during secondary infection (Diamond & Pierson, 2015). Spatial patterns in concurrent and/or sequential circulation of DENV1–4 should be considered as potentially important population-level risk factors for severe dengue illness as well (Figure 1)(Messina et al., 2014).

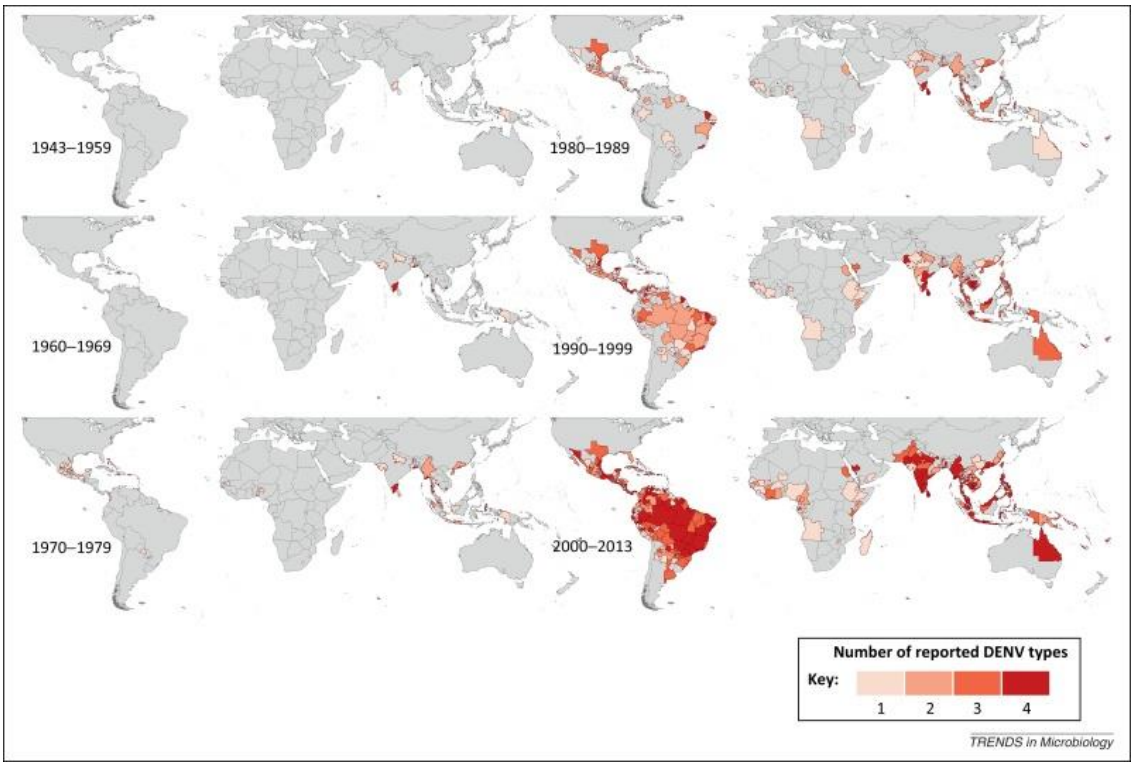


Figure 1. DENV co-circulation. Certain serotypes of DENV are prevalent in colored areas. Observation of potential increases in co-circulation of the four viruses, which may serve as a key indicator of progression toward hyperendemic transmission. (*Messina et al., 2014*)

Current control measures

At present, the major approach to control or prevent the transmission of DENV is to combat vector mosquitoes (WHO). Mosquitoes transmit the virus by first taking an infectious blood meal. Viruses within the blood are ingested in the mosquito midgut, infect and replicate in the midgut epithelial cells. As the progeny viruses are released into mosquito hemolymph they can reach and infect other organs, including the mosquito salivary glands. After 10–14 days post initial blood meal, viruses in the salivary glands can be inoculated to

another person through a subsequent blood meal, which completes the transmission cycle (Nedjadi et al., 2015).

Currently, vector control efforts aim to reduce mosquito population density by targeting the immature aquatic stages or the adult stages (Bowman et al., 2016). Control efforts involve environmental, chemical and biological management approaches. Environmental modification or sanitation improvements require a continuous effort. The effectiveness of these interventions is often impaired by interruption due to insufficient financing to maintain infrastructures (Anders & Hay, 2012). Additionally, the use of traditional chemical insecticides, unsurprisingly, apply an environmental selection for resistant mosquitoes (Lam, 2013). One innovation in biological management is highlighted with the *Wolbachia*-based control. *Ae. aegypti* mosquitoes stably infected with strains of the obligate intracellular bacterium *Wolbachia* are resistant to DENV infection and are being tested in field trials in Australian (Ferguson et al., 2015). Noticeably, these antiviral effects rely on a complex tripartite system (the host, *Wolbachia* and virus) with the potential of unpredictable co-evolution (Johnson, 2015). Other novel control mechanisms include the development and release of a genetically modified *Ae. aegypti* strain carrying specific genes that abolish transmission cycle (Lam, 2013). However, these efforts have not eliminated disease transmission in countries where dengue is endemic (Hermann et al., 2015).

To date, only one vaccine against dengue virus has been licensed. This vaccine consists of live-attenuated tetravalent chimeric dengue-yellow fever

vaccine strains. The DENV serotype-specific structural proteins are incorporated in a yellow fever virus 17D backbone (Weiskopf et al., 2014) (Nedjadi et al., 2015). Phase I and II trials conducted in adults in endemic and non-endemic regions have demonstrated a favorable safety profile and high immunogenicity. However, the vaccine was shown to be only effective in protecting against disease caused by DENV1 [61.2%], DENV3 [81.9%], DENV4 [90.0%] but not DENV2 after single-dose injection (Lam, 2013). There are other vaccine candidates in development, yet none of them have been tested in Phase III trials. In summary, an imperfect vaccine alone is obviously not enough to curb dengue transmission (Christofferson & Mores, 2015).

Molecular basis of DENV

DENV has a positive-sensed, single-stranded, capped RNA genome. The 10.7 Kb genome encodes 3 structural proteins (C, capsid protein; prM, precursor membrane protein; and E, envelope protein) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Zhang, Sun, & Rossmann, 2015). During translation of the viral genome (+ssRNA), a single polyprotein is produced, and sequentially processed by cellular and viral proteases, releasing several structural and non-structural proteins as illustrated by Figure 2. The corresponding function of each of those proteins is reviewed in Table 1.

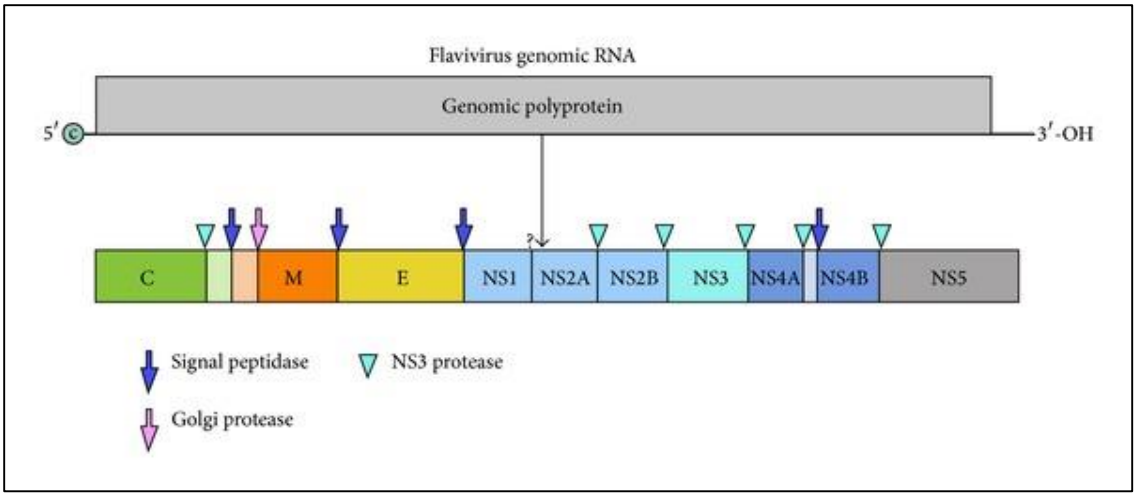


Figure 2. Flavivirus genomic RNA and its translation product.
(Hernandez, Brown, and Paredes 2014)

Table 1. DENV proteins and their functions
*(Adapted from Nedjadi et al., 2015; Baharuddin et al., 2014**;** Tomlinson, Malmstrom, & Watowich, 2009)*

DENV	**Viral proteins**	**Functions**
Structural proteins	Capsid (C)	• Interacts with the genomic RNA to promote packaging into immature virions
	Pre-membrane (prM)	• An M protein precursor that primarily prevents premature rearrangement of the E protein under the mildly acidic conditions of the trans-Golgi prior to virion release • In the trans-Golgi network, the prM protein is cleaved by cellular furin to allow M/E rearrangement to produce the mature virion.
	Envelope (E)	• Recognizes an unknown receptor on target host cells to induce viral uptake • Required for membrane fusion • Contains epitopes for neutralizing antibody
Non-structural proteins (NS)	NS1	• Involved in host immune response evasion
	NS2A	• Poorly defined
	NS2B	• Cofactor protein in the protease function of NS3

	NS3	• NTPase, helicase, and RTPase activities
	NS4A	• Involved in induction of membrane rearrangement and/or autophagy response to viral infection of host cells
	NS4B	• Anchor and target the replication complex to the endoplasmic reticulum (ER) membrane • Immune response antagonism
	NS5	• RNA dependent RNA polymerase (RdRp) • Methyltransferase • Guanylyltransferase for mRNA capping

DENV replication cycle

DENV genome is enclosed by a capsid shell surrounded by a lipid bilayer envelope with envelope proteins (prM/E heterodimers) on the outer surface. The virus attach to target cells via the interaction of E protein (53KDa) with a variety of cellular receptors, including glycosaminoglycans (heparin sulfate), C-type lectins (DC_SIGN [CD209] and the mannose receptor [CD206]), and immunomodulatory proteins (TIM/TAM receptors) (Diamond & Pierson, 2015). Upon attachment, receptor-mediated endocytosis is induced. The virus is internalized into the endosome, where proton-pumps on the endosomal membrane acidifying the endosome. In response to lowered pH, prM/E heterodimers arranged into a trimer undergo conformational changes to form "spike"-like structures (Figure 3) and expose a hydrophobic fusion loop of the E protein (Figure 3. Red stars). This hydrophobic fusion loop inserts into the endosomal membrane, initiating a bending bridge between the endosomal and the virus membrane, leading to membrane fusion. Hence, the nucleocapsid is

released into the cytoplasm (Urcuqui-Inchima, Patiño, Torres, Haenni, & Díaz, 2010). It is believed that the host membrane composition can play an important role in virus fusion (Rogers, Kent, & Rempe, 2015). Once in the host cytoplasm, the capsid thereby breaks apart, releasing the viral genome. The +ssRNA is translated, utilizing host machinery, into a single polyprotein. The polyprotein is further processed into structure proteins for assembly and non-structure proteins for viral genome replication. Genome replication occurs in virus–induced membrane invaginations of the ER, coupled with encapsidation (Fischl W. and Bartenschlager R.; Apte-Sengupta, S. et al.; Chatel-Chaix & Bartenschlager). Once viral RNA associate with multiple copies of the C protein, viral genomes are packaged into nucleocapsids (NC), acquiring the prM/E heterodimers (Figure 2.A) containing ER membrane as immature particles into the lumen (Acosta, Kumar, & Bartenschlager, 2014). Immature particles will hijack the cellular canonical secretory pathway to egress (Chatel-Chaix & Bartenschlager). The slightly acidic pH (~5.8-6.0) of the trans-Golgi network (TGN) triggers dissociation of the prM/E heterodimers (Keelapang et al., 2004) (Figure 3.B). Viral particles mature further when furin (a trans-Golgi resident enzyme) cleaves the prM (approximately 21 KDa) into the pr peptide and the M proteins (8kDa) (Smit, Moesker, Rodenhuis-Zybert, & Wilschut, 2011) (Figure 3.C). The pr peptide remains associated with the virion until it has been released from the cell (Figure 3.D). Both the prM protein and the pr peptide are believed to act as chaperones stabilizing the E protein to prevent a premature conformational rearrangement of the virion throughout the secretory pathway (Fischl W. and Bartenschlager R., 2011; Rodenhuis-Zybert et al., 2010).

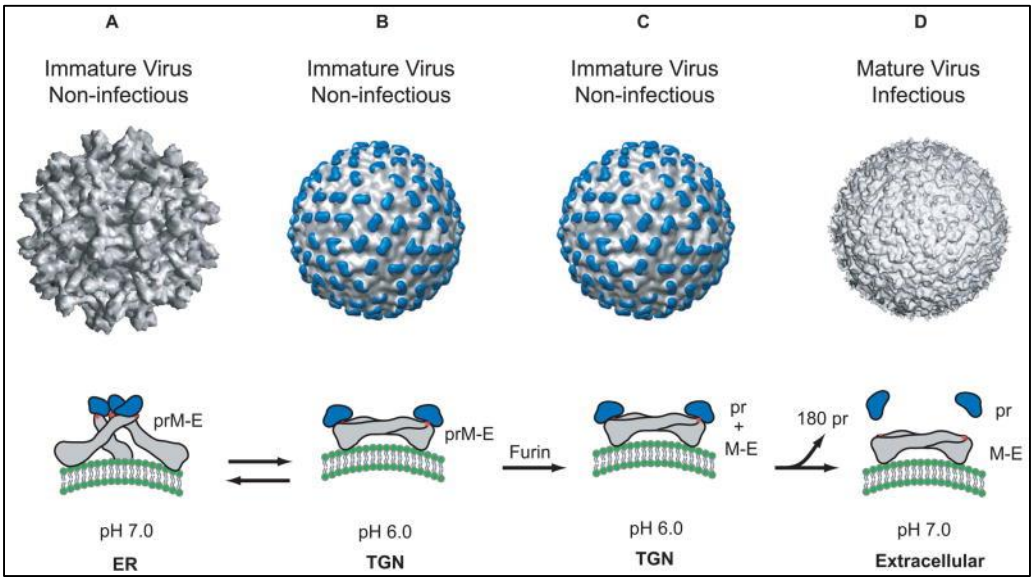


Figure 3. Structure of the dengue virion and conformations of the E protein during its maturation. In supernatants of infected cells, the virus is found either as a mature or immature particle with a diameter of about 50 nm and 60 nm, respectively *(Perera & Kuhn, 2008)*.

Additionally, change from smooth to "spiky" conformation of virion (specifically DENV2) can not only be triggered by low pH, but by incubation at physiological temperature (37°C) (Zhang et al., 2015). Several studies suggest naturally occurring oscillation among ensemble of different structures, a phenomenon referred to as virus "breathing" (Guzman & Harris, 2015) (Dowd, DeMaso, & Pierson, 2015) (Zhang et al., 2015). The "breathing" structure might be able to explain the difficulty in deciphering mechanisms for ADE and the heterogeneity of pathogenesis.

Antiviral target and development

Recent advances in drug discovery in combination with high-throughput screening (HTS) and structure-based drug design (SDD) have allowed the identification of drugable targets within the DENV replication cycle (Tomlinson et al., 2009) (Teixeira et al., 2014). Traditional direct-acting antiviral agents (DAA) target the enzymatic replication proteins including the protease-helicase (NS2B-NS3) (Luo, Vasudevan, & Lescar, 2015), components of replication complex (NS4B) (Xie, Zou, Wang, & Shi, 2015), and the viral polymerase/RdRp (NS5) (Malet et al., 2008). Loss of function for any of these proteins will block virus propagation. Controversially, the suboptimal specificity of enzyme inhibitors or nucleoside analogs sometimes may occur at a risk for selection-driven evolution of emergent drug-resistant viruses, which is further facilitated by the error-prone nature of RNA-dependent RNA polymerase (RdRp). To prevent the outgrowth of resistance, a novel strategy has been introduced to target the hybridization of the targeted drug-susceptible and drug-resistant core proteins, defined as "dominant drug targets" (Mateo, Nagamine, & Kirkegaard, 2015.).

In the light of molecular docking and stereochemistry studies, one can also design, or screen for, inhibitors that target critical regions of structural proteins, including the E protein β-OG pocket (Tambunan, Zahroh, Parikesit, Idrus, & Kerami, 2015) and the E protein stem region (Schmidt, Yang, & Harrison, 2010) (Figure 4.). By targeting the E protein, these inhibitors also inhibit other replication steps involving the E protein (i.e. endosomal escape), rather than prevention of entry itself. The E protein stem region has a conserved amino acid sequence among all DENV serotypes and other

flaviviruses, providing another advantage as a drug target (Botting & Kuhn, 2012).

Drugs are also designed to target host factors essential for efficient viral replication. Examples mainly include glucosidases (Chang et al., 2012; Courageot, Frenkiel, Dos Santos, Deubel, & Desprès, 2000), kinases (de Wispelaere, LaCroix, & Yang, 2013; Smith et al., 2014), and the cholesterol biosynthesis pathway (Lee, Lin, Liao, & Lin, 2008). For example, Celgosivir, a clinically approved prodrug of castanos-permine, a product of the Moreton Bay chestnut tree (*Castanospermum australae*) proved to be an α-glucosidase inhibitor that prevent glycosylation of the NS1 protein (Botting & Kuhn, 2012). It has also been shown that α-glucosidase inhibitors strongly affects productive folding pathways of the prM and the E protein (Courageot et al., 2000). Unfortunately, the study failed to demonstrate clinical benefit in Phase 1b trails (Schapira, 2004).

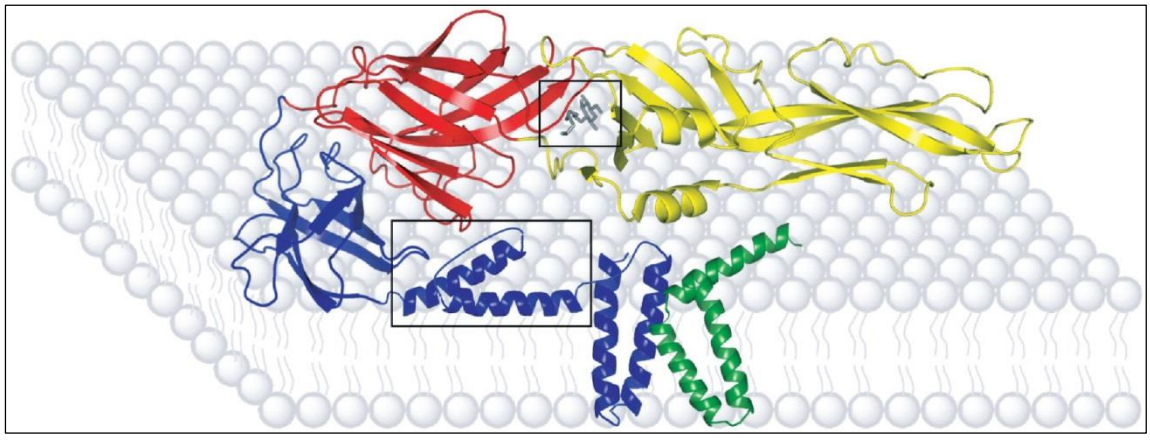


Figure 4. Sites of DENV E protein inhibition. *(Botting & Kuhn, 2012.)*
The E protein domains are indicated by color; domain I in red, domain II in yellow and domain III in blue. The relative position of the M protein is indicated by green shading. The β-OG pocket and stem region (boxed) are common sites

of E protein targeting. The crystal structure of the E-protein indicates a ligand-binding pocket that is filled with a detergent molecule, η-octyl-β-D-glucoside (β-OG). This finding led to a number of docking studies to screening compounds for inhibition of E protein via the β-OG pocket*(Baharuddin et al., 2014)*.

Isolation of *Chromobacterium. sp Panama* (*Csp_P*)

The symbiotic relationship between gut flora and their host organism has been widely studied (Hill, Sharma, Shouche, & Severson, 2014). The importance of this relationship, lead to an interest in the mosquito microbiota and its influence on the transmission of vector-borne pathogens. The potential application of microbes to control the transmission of vector-borne pathogens has been highlighted by several studies in the past decade. Based on the rationale that pathogens and microbes interact naturally in the mosquito midgut, an interest in understanding how bacteria influence the outcome of infection emerged. Previously, researchers in the Dimopoulos group isolated a Gram-negative, soil bacterium, *Chromobacterium* (*Csp_P*), from the midgut of *Ae.aegypti* mosquito in DENV-endemic areas in Panama (Figure 5.). When *Csp_P* is fed to mosquitoes prior to DENV infection, it can effectively colonize the mosquito midgut and significantly inhibit virus infection (Figure 6.) (Ramirez, Jose Luis et al.). Possible mechanisms underlying the compromised DENV infection are unknown, however, colonization with *Csp_P* induces mosquito innate immune system genes. In addition to the potential role of immune modulation, *Csp_P* appears to inhibit DENV replication *in vitro*, indicating a possible production of *Csp_P*-derived anti-DENV factors with transmission-blocking and therapeutic potential (Ramirez et al., 2014).

These findings lead to a couple of intriguing questions: what are the anti-DENV factors being produced by *Csp_P* and how do they inhibit DENV replication *in vitro*. Here we described a study aiming to identify *Csp_P*-derived anti-DENV factors by using variety of classical methodologies. The experiments revealed that the anti-DENV factor is very likely to be bacterial secreted proteins that form a multiprotein complex. We also investigated the step of the DENV replication being affected by *Csp_P*-derived anti-DENV factors, and the mechanism of inhibition. Our *in vitro* and cell-free analysis favor a mechanistic model were *Csp_P*-derived anti-DENV factors inhibit DENV attachment to host cells by disrupting the structural integrity of DENV virions.

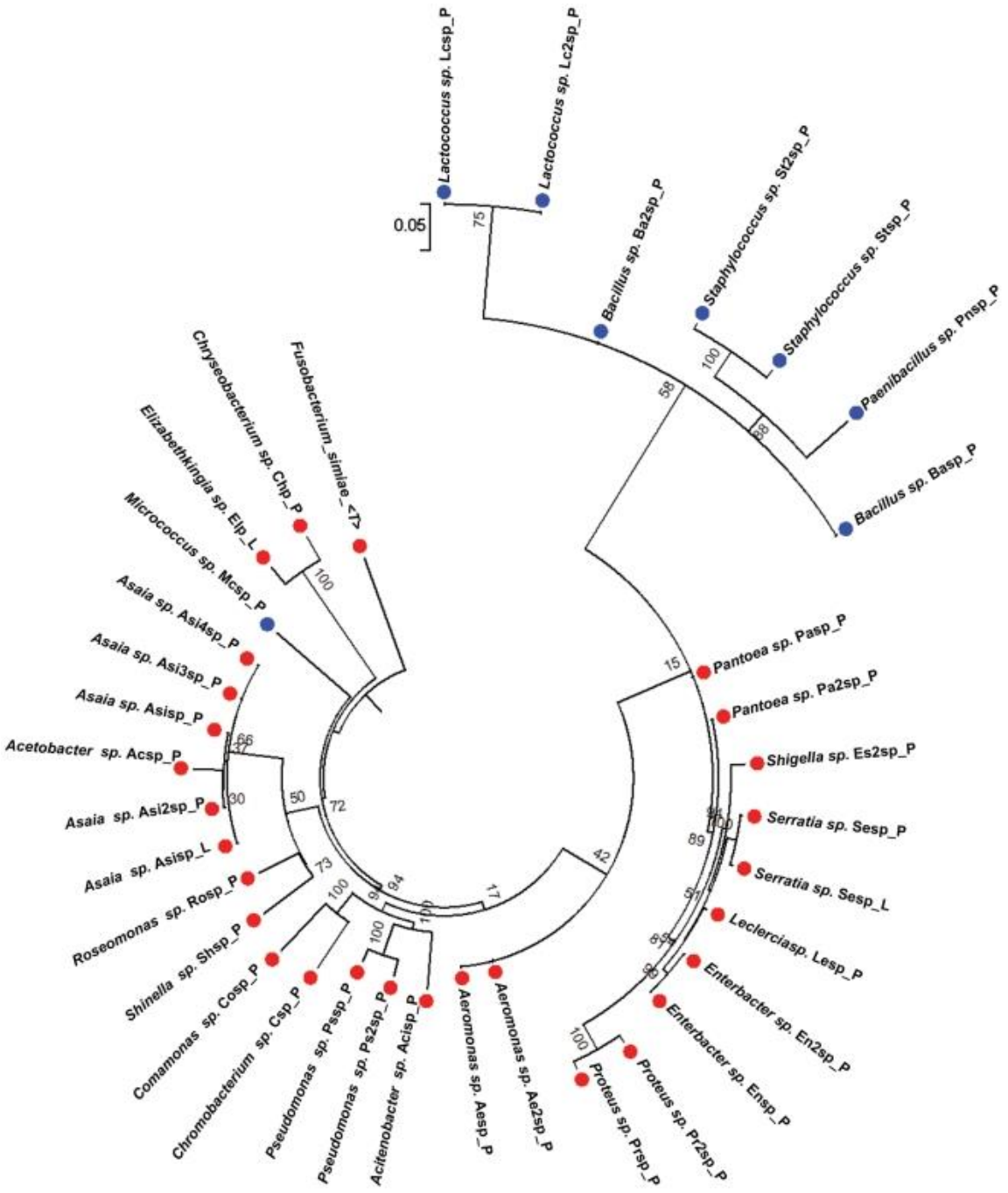


Figure 5. Phylogenetic tree of the field and laboratory-reared *Ae. aegypti* cultivable midgut microbiota. (*Ramirez, Jose Luis et al.*)
Red dots: Gram-negative, blue dots: Gram-positive.

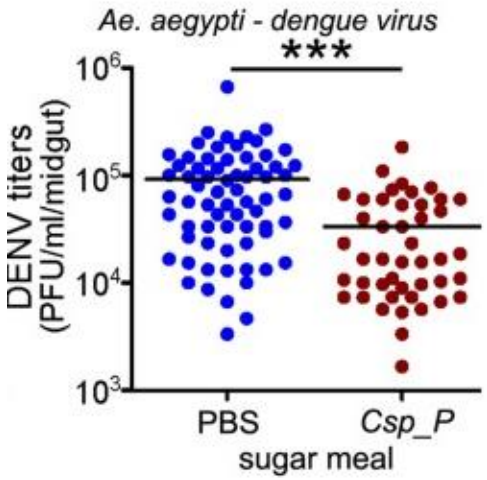


Figure 6. *Csp_P* reduces mosquitoes' susceptibility to malaria and dengue infection. Antibiotic-treated adults were allowed to feed for 24 h on 1.5% sucrose containing *Csp_P* liquid culture at a final concentration of ~10^6 CFU/ml for *Ae. aegypti*. After introduction of *Csp_P* via the sugar meal, *Ae. aegypti* mosquitoes were given a blood meal that contained dengue virus. At 7 days after infection, midguts were dissected. Dengue virus titers were assayed in dengue-infected *Ae. aegypti* females by conducting standard plaque assays. Experiment were replicated at least three times with final samples sizes: PBS=68, *Csp_P*=45. Differences between treatments were assessed by Mann-Whitney test (*, $p<0.05$; ***, $p<0.001$). *(Adapted from Ramirez et al.)*

MATERIALS AND METHODS

Cell lines

Baby hamster kidney cells (BHK-21 cells) were grown at 37°C in Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum, penicillin, streptomycin and plasmocin. *Aedes albopictus* clone C6/36 cells (C6/36 cells) were grown at 32°C in Minimum essential medium with non-essential amino acids and additives as above.

Virus Stock

C6-36 cells were used to propagate DENV-2 New Guinea C strain. C6-36 cells were incubated with DENV2 for 15 minutes on the shaker at room temperature following by 45 minutes at 32°C. After 6 days post infection (dpi), cell culture medium as well as cell lysis was collected. Supernatant containing virus was clarified by centrifugation at 1,500 – 2,000 g for 10 minutes. Virus stock was aliquoted and stored at -80°C for later use.

Thermostability assay

Csp_P supernatant was obtained by 72 hours *Csp_P* planktonic culture filtered through 0.22um membrane filter. Supernatant was aliquot into 100ul per tube and incubated in the dry bath incubator at 37, 50, 70, 99 °C respectively for 1 hour. Supernatant after heat treatment were then evaluated for their anti-dengue activities by plaque reducing assay described as following.

Protein extract preparation

Csp_P supernatant was prepared as described above. Solid ammonium sulfate (AS) was slowly added to the supernatant to reach 30% saturation. After rapid stirring at 4°C for 1 hour, proteins (AS30%) were pellet down by centrifugation

at 10,000 *g* for about 20 minutes. The supernatant was carefully poured off and its volume was determined. Again solid AS was slowly added to the supernatant to reach 70% saturation. After rapid stirring at 4°C for 1 hour, proteins (AS70%) were pellet down by centrifugation at 10,000 g for about 20 minutes. Protein pellet (AS70%) was gently resuspent in 0.1M Tris-HCl pH 7.2 buffer with protease inhibitors- EDTA free (Burgess, 2009). Protein concentration was estimated by Nanodrop at 280nm. Protein resuspension was fractionated sequentially by ultrafiltration (UF) with 100, 50, 30, 10, 3 kDa membrane molecular weight cut off (MWCO). Each fraction was collected after centrifugation at 5000*g* for 10-20 mins. Protein concentration was roughly determined by Nanodrop at 280 nm. Recovery rate was calculated (>90%). Protein components of each fraction was visualized by SDS-PAGE gel with silver staining.

Protein sequencing

The 50-100kDa fraction of *Csp_P* AS70% resuspension was separated further by SDS-PAGE 4- 20% Tris-Glycine gel. The protein band of interest was identified by comparison with other fractions. Multiple bands of interest by running parallel sample were excised and poured from a Colloidal Blue Staining Kit (Invitrogen) stained gel. Gel pieces were washed in methanol and sent to the Johns Hopkins School of Medicine Mass Spectrometry and Proteomics Core Facility for identification using iTRAQ as described previously in detail (Pike A. et al.; 2015). MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.5.1). Mascot was set up to search the 160105_Chromobacterium_160501 database (unknown version, 91996 entries)

assuming the digestion enzyme trypsin. Scaffold (version Scaffold_4.4.8, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 34.0% probability to achieve an FDR less than 1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Amino acids sequences of identified proteins were further searched against *Chromobacterium sp. Panama* (*Csp_P*) database for unique hits.

Plaque reduction inhibition assay

BHK-21 cells or C6-36 cells were seeded at ~8.4 x 10^5 cells/well (24-well plate) and then incubated at 37 or 32 °C overnight to reach 80~90% confluence. Prior to infection, 50 ul of DENV2 stock (NGC strain) was pre-incubated with each sample (1v:1v) at room temperature. After 1 hr, DENV2 and sample mixture was serially diluted (1:10) in DMEM medium. Cells were co-infected with DENV2-sample dilution for 15min at room temperature on the shaker followed by 45min at 32°C. ~1ml of overlay medium was added to each well. The plates were incubated for 5-6 days at 32°C under 5% CO_2. BHK-21 cells were fixed and stained with 1% crystal violet in methanol/acetone (1v:1v) solution. C6-36 cells were fixed with methanol/acetone (1v:1v) and blocked with 5% skim milk in PBS for 10 mins. Fixed C6-36 cells were incubated with mouse anti-DENV2 4G2 antibody from ATCC (1:1000-1:2000) for 2 hrs at room temperature. After washed with PBS twice, C6-36 cells were incubated with goat anti-mouse IgG-

HRP (1:1500) for 1.5 hrs at room temperature, following two rounds of PBS wash. Air dry the plate and add True Blue Peroxidase Substrate (KPL) to react for 5 mins on the shaker. The number of plaque forming units (PFU) per ml was determined.

Immunofluorescence assay

BHK 21 cells were seeded in 24-wells plate with cover slip in each well. After 72hr growth at 37 °C, cells were infected with DENV-2 at a MOI of 10 in the presence of absence of *Csp_P* proteins for 1hr at 4 °C. Unbound virus are removed by washing 3 times with ice-cold PBS. Cells were fixed with 4% PFA for 1 hr at 4 °C. Fixed cells were blocked with 0.5% BSA in PBS for 1 hr at room temperature, washed twice with PBS and incubated with m4G2 anti-DENV2 antibody (ATCC) for 2 hrs at room temperature. Alexa Fluor 568 goat anti-mouse IgG (Invitrogen) was used as secondary antibody. Beta-actin was stained with Alexa Fluor 488 Phalloidin (Invitrogen). Nuclei were stained with DAPI. Samples were mounted by ProLong Gold antifade reagent. Fluorescence analysis was performed on a Leica DM 2500 fluorescence microscopy (Vervaeke et al., 2013).

Time course studies on the anti-DENV activity

BHK-21 cells were seeded at ~8.4 x 10^5 cells/well (24-well plate) and then incubated at 37 overnight to reach 80~90% confluence. *Csp_P* supernatant were added under the following conditions:

Pre-infection treatment: BHK-21 cells were treated with serial dilutions (1:10) of *Csp_P* supernatant (100ul/well). After 1 hour incubation at 37 °C, the cells

were washed twice with PBS and infected with 100ul/well of DENV2 at the same serious dilution for 1 hour at 37 °C. Overlay medium was added and the viral titer was determined 5 days post infection using plaque reduction inhibition assay.

Co-infection treatment: BHK-21 cells were treated with serial dilutions (1:10) of *Csp_P* supernatant and DENV2 (1v:1v) in 100ul/well. After 1 hour incubation at 37 °C, overlay medium was added and the viral titer was determined 5 days post infection using plaque reduction inhibition assay.

Post-infection treatment: BHK-21 cells were infected with serial dilutions (1:10) of DENV2 (100ul/well) for 1 hour. The cells were washed with PBS and treated with Csp_P supernatant (100ul/well) at the same serious dilution for 1 hour at 37°C. Overlay medium was added and the viral titer was determined 5 days post infection using plaque reduction inhibition assay (Schmidt et al., 2012).

Virus attachment assay

Pre-attachment: BHK-21 cells in 24-well plates and reagents were cooled to 4°C. Virus and *Csp_P* extract were incubated at 4°C for 1h, then transferred to chilled BHK-21 cells and incubated for another 1h at 4 °C. After incubation, cell monolayers were washed 3X with cold PBS, overlay medium was added and cells were incubated at 37 °C for 5 days before fixing and staining for plaque forming unit.

Post-attachment: BHK-21 cells in 24-well plates and reagents were cooled to 4°C. Virus was added to cells and allowed to bind for 1h at 4°C. Unbound virus was washed off by washing twice with cold PBS. *Csp_P* extract was then added

to virus bound cells and incubated for 1h at 4°C. Following incubation, cell monolayers were washed once with cold PBS. Overlay medium was added and cells were incubated at 37 °C for 5 days before fixing and staining for plaque forming unit (Fibriansah et al., 2014).

Cell viability assay by trypan blue staining

BHK-21 cell suspension ($1x10^5$ to $2x10^5$ cells/ml) was prepared in DMEM without FBS. 50ul of suspension was put in each Eppendorf tube, mixed with 10ul of sample or control (10% DMSO). Tubes were incubated at 37°C for 1 hour. 12ul of 0.4% Trypan Blue Stain (Invitrogen) was added to each tube and incubate for 5 minutes. 10ul of cell suspension from each tube was harvested and added to hemacytometer (Thermos). Cells were counted in the microscope under a 20X objective.

Western blot analysis for Csp_P proteins treatment

Solutions of sample and control were prepared as follows:

A: 50ul of DENV2 stock + 50ul of 0.1M Tris-HCl buffer;

B: 50ul of DENV2 stock + 50ul of *Csp_P* protein suspension;

C: 50ul of DENV2 stock + heat inactivated *Csp_P* protein suspension;

D: 50ul of MEM medium + 50ul of *Csp_P* protein suspension.

After 1 hour incubation at room temperature, each solutions were added to individual filtration tubes (MWCO= 100kDa) filled with 4ml of 1M Tris-HCl pH7.2 buffer (higher salt concentration to reduce non-specific binding). Centrifuged for 15 mins at 5,000 *g*. Concentrated sample and control after treatment were collected and applied on SDS-4-20% polyacrylamide gels.

Protein bands were later on transferred to a nitrocellulose membrane, following overnight blocking at 4°C in 5% silk milk-PBST. Dengue2 E proteins were probed by anti-E rabbit polyclonal antibodies (1: 2,000 dilution and anti-rabbit HRP (1:20,000 dilution) was added for 1 hour incubation. 3 times of washing with PBST between each step. Signals were detected with Amersham ECL kit.

Statistical Analysis

Data from plaque assays were analyzed using the GraphPad Prism statistical software package (Prism 5; GraphPad Software, Inc., San Diego, CA). Unpaired t-test (two-tailed) was conducted on the mean value of triplicates from least two individual biological replicates. Statistical significance numerated by p-value is indicated with asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

RESULTS

Effect of Csp_P culture supernatant on in vitro DENV replication

Previously, Ramirez *et al.* have shown the *in vitro* anti-DENV activity of *Csp_P* liquid culture. In their study, *Csp_P* bacterial preparation (unfiltered) was mixed with DENV2 virus stock (1v: 1v) and incubated at room temperature for 45 min. This mixture was then filtered before anti-DENV measurement. Here we assessed if *Csp_P* culture supernatant (*Csp_P* sup) without live bacteria could still inhibit DENV2 replication. *Csp_P* bacterial preparation (filtered) was mixed with DENV2 virus stock (1v:1v) and incubated at the same conditions. DENV2 replication in both BHK21 cells (mammalian) and C6/36 cells (mosquito) was inhibited (*P* value <0.0001 in BHK21 cells, *P* value=0.0003 in C6/36 cells) compared to the LB control (Figure.7 upper). A similar level of anti-DENV2 activity, in both cell lines, indicates that the target of the *Csp_P*-derived factor(s) could be viral proteins, RNA or host factors conserved across species (Haridas et al., 2013). In crystal violet staining of *Csp_P* culture supernatant treated BHK21 cells did not result in a significant cell detachment, while virus infection was inhibited (Figure 7. lower left). These results suggest that, under the inhibitory concentration of *Csp_P* culture supernatant, the viability of BHK21 cells is not significantly affected. However, additional experiments are required to study the cytotoxicity profile.

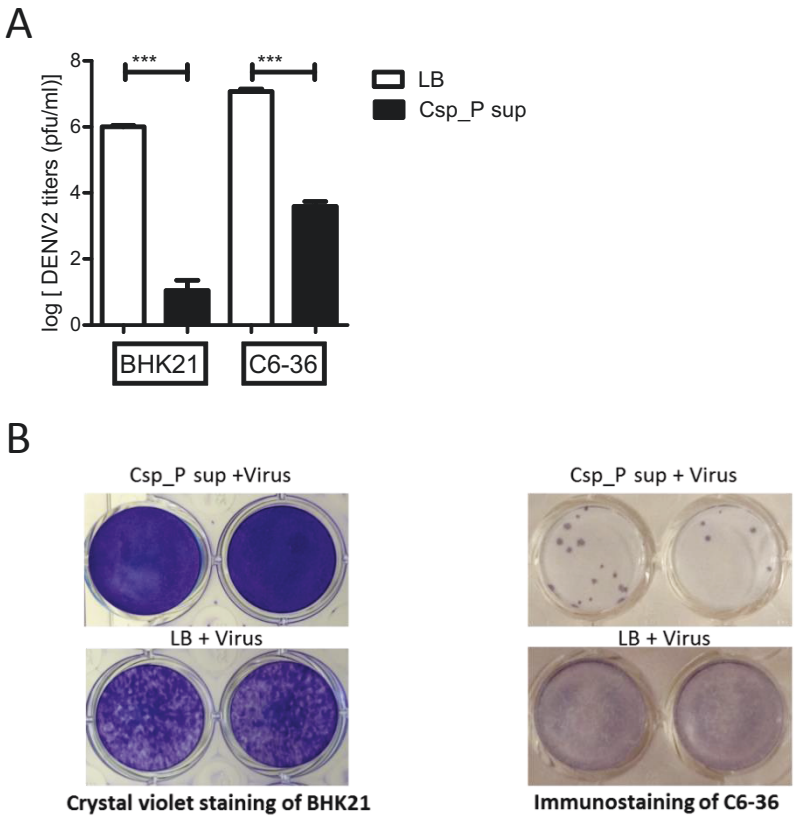


Figure 7. *Csp_P* supernatant inhibits DENV2 replication in BHK21 cells and C6-36 cells. Prior to infection, DENV2 stock was incubated with *Csp_P* sup for 1 hour at room temperature. BHK-21 cells were then infected with *Csp_P*-DENV2 (at MOI=0.01) for 5 days at 37° C and stained with crystal violet solution (each empty plaque represent an infectious colony). Similar experiments were done using C6-36 cells, except DENV2 infectious colonies in C6-36 cells (MOI=0.01) were immunostained with anti-DENV2 m4G2 antibody (empty space represent non-infectious cells). (A) Strong inhibition of DENV2 conferred by *Csp_P* supernatant was observed in both BHK21 cells ($P < 0.0001$) and C6/36 cells ($P = 0.0003$). (B) Representative plaque assay is shown. Error bars represent the standard error of the mean from 3 biological replicates with technical triplicates. Statistical analysis was performed using unpaired t test (***$P < 0.001$).

The *Csp_P* –produced anti-DENV has protein-like properties

As shown above, *Csp_P* culture supernatant is able to inhibit DENV replication independently from the presence of live bacteria. To isolate anti-

DENV factors from this supernatant, we first assayed the anti-DENV activity in different fractions of the *Csp_P* supernatant. We observed appreciable anti-DENV activity of the protein fraction (Asp70%50-100K), which precipitated by ammonium sulfate at a saturation of 70% (Asp70%), with molecular weights ranging from 50 to 100kDa.

Thermal stability of Csp_P supernatant

There are a variety of molecules in a bacterial culture supernatant, such as proteins, lipids, polysaccharides, nucleic acids and other small molecules (secondary metabolites). Among them, secondary metabolites often harbor aromatic rings that stabilize their structures at high temperatures. On the contrary, macromolecules (especially proteins) are unstable at temperatures between 40-80°C (Bischof & He, 2005). To determine the thermostability of anti-DENV factors, samples of *Csp_P* culture supernatant were heat-treated for 1 hour at 37, 50, 70, and 99 °C, and then pre-incubated with DENV for 1 hour prior to virus titer determination through plaque assays. As interpreted by the observed changes in DENV titers, the anti-DENV factors within the *Csp_P* culture supernatant were stable up to 50°C, but were inactivated at a 70°C treatment for 1h (Figure 8). This result indicates a heat-sensitive factor-likely a protein or protein complex – is responsible for the anti-DENV activity.

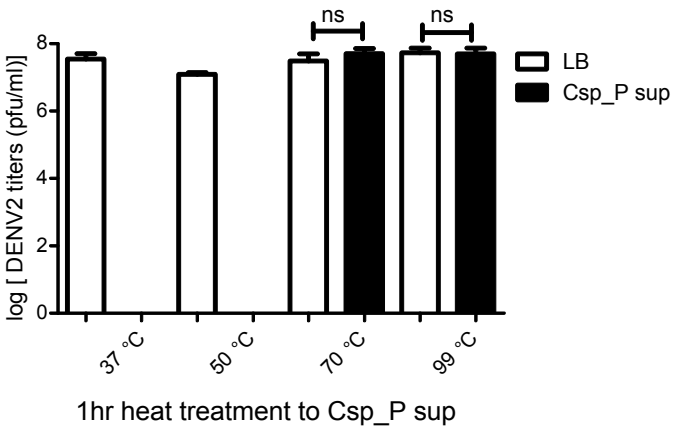


Figure 8. Anti-DENV activity of *Csp_P* supernatant is destroyed at temperatures higher than 70 °C. Samples of *Csp_P* sup and LB control were heat-treated for 1 hour at different temperatures (37, 50, 70, 99°C). DENV2 stocks were pre-incubated with heat-treated *Csp_P* sup and LB control for 1 hour. Anti-DENV activity of each heat-treated sample of *Csp_P* sup was tested by plaque assay on BHK-21 cells, and compared to that of paralleled LB control with the same treatment. Upon 1 hour heat-treatment at over 70°C, *Csp_P* sup confers no significant inhibition to DENV2. No plaque was detected in plaque assays using heat-treated (at 37 and 50°C) *Csp_P* sup, thus no statistical analysis could be done with these two groups. Error bars represent the standard error of the mean from 3 biological replicates with technical triplicates. Statistical analysis was performed using unpaired t test (ns, not significant).

- **Bioseparations of Csp_P-derived anti-DENV factors**

To verify the protein-like property of anti-DENV factors, we measured the anti-DENV activity of *Csp_P* a secreted protein extract. Our strategy for extracting proteins from the *Csp_P* culture supernatant was precipitation: the solubility of proteins decreases with increased concentration of the precipitant in the aqueous phase. As a classical precipitant, ammonium sulfate (AS) is stabilizing protein structures, is soluble and relatively inexpensive (Burgess, 2009). Although we were also able to extract *Csp_P* proteins by TCA-acetone (another classical precipitation method), we noticed an irreversible denaturation of *Csp_P* proteins when resuspended in 0.1M Tris-HCl pH7.2 buffer (compared

to AS precipitation). Upon AS precipitation at a saturation of 70%, *Csp_P* proteins were precipitated, pelleted, resuspended in a 0.1M Tris-HCl pH7.2 buffer and then desalted by ultracentrifuge. The remaining *Csp_P* supernatant was desalted and concentrated by ultra-centrifuge. After pre-incubation of DENV2 with the pellet resuspension and supernatant, the anti-DENV activity of both fractions was tested by plaque assays. We observed a dramatic anti-DENV activity in *Csp_P* proteins pellet (Figure 9. pellet) but not in the supernatant (Figure 9. sup). The anti-DENV activity of *Csp_P* proteins was confirmed by a dose-response relationship between the concentrations of the AS 70% protein suspension (ASp70%) and its anti-DENV activity measured by plaque assay (Figure 10).

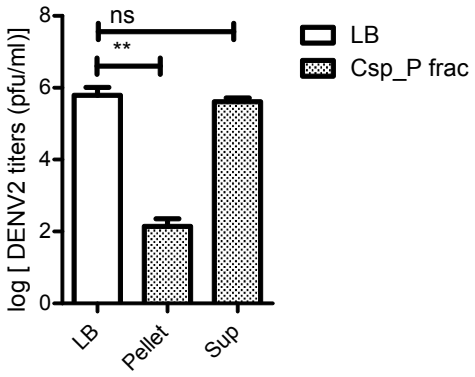


Figure 9. The anti-DENV activity of *Csp_P* proteins. *Csp_P* proteins (Pellet) were precipitated by ammonium sulfate (AS) at the saturation of 70% and resuspent in 0.1M Tris-HCl pH7.2 buffer. The supernatant left behind (Sup) was desalted and concentrated. DENV2 stocks were pre-incubated with either fraction (Pellet/ Sup) or LB control for 1 hour. Anti-DENV activity of each of *Csp_P* fractions was tested by plaque assay on BHK-21 cells and was compared to that of LB control. Significant inhibitory effect on DENV2 was observed from *Csp_P* protein pellet ($P = 0.0083$) rather than sup, measured by plaque assay on BHK21 cells. Error bars represent the standard error of the mean from 3

biological replicates with technical triplicates. Statistical analysis was performed using unpaired t test (***$P < 0.001$; ns, not significant).

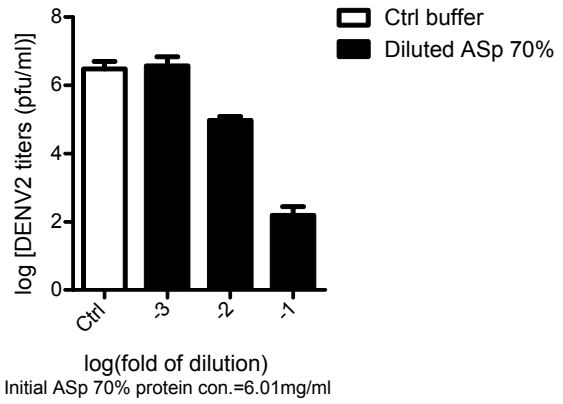


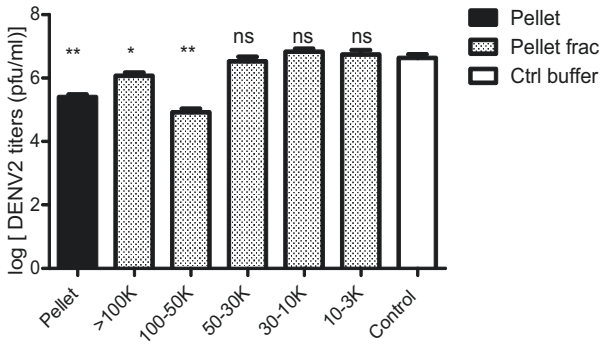
Figure 10. Dose-dependent anti-DENV activity of *Csp_P* proteins (ASp 70%). Serially diluted *Csp_P* proteins (resuspension of *Csp_P* proteins pellet, 6.01mg/ml quantified by BCA assay) were pre-incubated with DENV2 stocks, anti-DENV activities were measured by plaque assay on BHK21 cells. As fold of dilution increase from 1:10 to 1:100, anti-DENV activity conferred by *Csp_P* proteins (Asp70%) was gradually decreased. Error bars, represent the standard error of the mean from 3 biological replicates with technical triplicates. 0.1M Tris-HCl pH 7.2 buffer was used as control and for series dilution.

Assessing the size of the anti-DENV factor

Further fractionation of the AS70% pellet was carried out using ultrafiltration with different molecular weight cut off (MWCO) membranes, ranging from 100kDa to 3kDa. The resuspended AS70% pellet was thereby divided into 5 fractions: >100kDa, 100-50kDa, 50-30kDa, 30-10kDa and 10-3kDa fraction. The 100-50kDa fraction contained the anti-DENV activity of the AS70% pellet (Figure 11.A) ($P = 0.0015$) compared to that of the AS70% pellet ($P = 0.0021$). However, no difference in protein composition between the fractions was observed by 1D SDS-PAGE analysis. In fact, smaller proteins (with mass

lower than the membrane MWCO) were also enriched in each fraction along with target proteins (with masses higher than membrane MWCO) (Figure 11.B). One possible reason could be protein-protein interactions that naturally exist. It could also be an artifact of ultrafiltration: the retained larger proteins creating an extra layer that retained the smaller proteins. Current results are however supporting the hypothesis that observed anti-DENV activity is associated with a secreted multiprotein complex derived from *Csp_P*, within the molecular weight range of 50-100kDa.

A



B

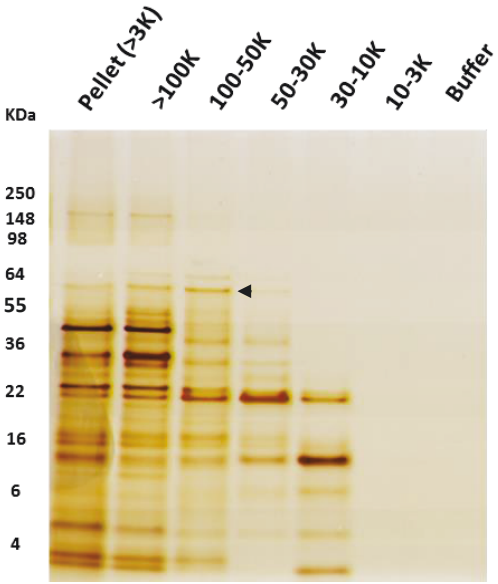


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Figure 11. *Csp_P* secreted proteins in 50 -100KDa are associated with anti-DENV activity. (A) *Csp_P* proteins extracted from ammonium sulfate precipitation (Pellet) was further separated into 5 fractions with different protein mass. Each fraction was adjusted to approximately the same protein concentration (determined by BCA assay) for further analysis. Anti-DENV activity of each fraction was tested by plaque assay on BHK21 cells. Within 5 fractions, only >100kDa (P = 0.0154) and 100-50kDa (P = 0.0015) fraction restored anti-DENV activity from ASp70% pellet (P = 0.0021), compared to buffer control respectively. DENV2 preincubated with 100-50K fraction [(8.3 ± 2.6) $x10^4$ pfu/ml] has lower viral titers compared to one preincubated with ASp70% pellet [(2.5 ± 0.53) $x10^5$ pfu/ml]. Error bars represent the standard error of the mean from 3 biological replicates with technical triplicates. Statistical analysis was performed using unpaired t test (*P < 0.1; **P < 0.01; ***P < 0.001; ns, not significant). (B) ASp 70% pellet and its 5 fractions were subjected to SDS-PAGE gel and silver stained. Arrow: hypothesized proteins associated with anti-DENV activity.

Proteomic analysis of *Csp_P* secreted proteins

Only one major protein band (Figure 11B, arrow) within the 100-50 kDa molecular weight was found in 100-50 kDa fraction by silver-stained SDS-PAGE analysis. Hence, we hypothesized that this protein band (Figure 11B, arrow) would be associated with most of the anti-DENV activity. We performed proteomic analysis of this protein band (~55kDa). iTRAQ labeling methodology was used in LC/MS/MS to fragment and analyze its potential identity. We identified 557 peptide spectrums that matched to 370 peptides that, in turn, mapped to 45 proteins using the 160105_Chromobacterium_160501 database (including multiple strains). These proteins were assigned putative names and functions through a BLASTp analysis against the *Chromobacterium Csp_P* genome, yielding 33 unique proteins (Table 2). After applying two additional filters; molecular weight between 50 to 64 kDa and the presence of signal peptides (analyzed by signal P 4.1)/non-classically secreted motif (predicted by

SecretomeP 2.0a), we finally obtained the following 7 Csp_p protein candidates: bifunctional metallophosphatase 5-nucleotidase, peptidase m4, phopq-activated pathogenicity related family protein, chitinase, tail protein, pkd domain containing protein, and fad-liked oxidase (Table 2. shading). Further experiments, either using mutagenesis or overexpressing protein candidates using a heterologous system, are required to confirm our proteomic analysis.

Table 2. Candidate protein genes in *Chromobacterium. Csp_P* identified by LC/MS/MS.

Seq Name	Description	e-Value	MW (kDa)	GO Names list
Csp_P_0011	peptide abc transporter substrate-binding protein	0.0E0	59	C:ATP-binding cassette (ABC) transporter complex; P:transmembrane transport
Csp_P_0105	glycosyl hydrolase family protein	0.0E0	73	F:beta-N-acetylhexosaminidase activity; P:globoside metabolic process; P:carbohydrate metabolic process; P:glycosaminoglycan catabolic process; P:amino sugar metabolic process
Csp_P_0263	arginine deiminase	0.0E0	44	C:cytoplasm; F:arginine deiminase activity; P:proline metabolic process; P:protein citrullination; P:arginine catabolic process to ornithine
Csp_P_0314	porin gram-negative type	4.2E-178	41	C:membrane; F:porin activity; P:transmembrane transport
Csp_P_0428	oligopeptidase a	0.0E0	76	F:metalloendopeptidase activity; P:proteolysis
Csp_P_0576	hypothetical protein	6.5E-111	21	C:integral component of membrane
Csp_P_0665	porin signal peptide protein	0.0E0	39	C:membrane; F:porin activity; P:transmembrane transport
Csp_P_0922	dihydrolipoamide dehydrogenase	0.0E0	50	C:cell; F:dihydrolipoyl dehydrogenase activity; F:flavin adenine dinucleotide binding; P:gluconeogenesis; P:glycolytic process; P:tricarboxylic acid cycle; P:obsolete electron transport; P:glycine metabolic process; P:L-serine metabolic process; P:threonine metabolic process; P:cell redox homeostasis

Csp_P_1055	fad-linked oxidase	0.0E0	59	F:UDP-N-acetylmuramate dehydrogenase activity; F:flavin adenine dinucleotide binding; P:amino sugar metabolic process; P:oxidation-reduction process
Csp_P_1074	porin	0.0E0	41	C:membrane; F:porin activity; P:transmembrane transport
Csp_P_1173	glutaryl-7-aminocephalosporanic-acid acylase	0.0E0	80	F:hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides; P:nitrogen compound metabolic process; P:antibiotic biosynthetic process
Csp_P_1811	tail sheath protein	0.0E0	49	-
Csp_P_1858	bifunctional metallophosphatase 5 -nucleotidase	0.0E0	59	F:5'-nucleotidase activity; F:UDP-sugar diphosphatase activity; P:protein dephosphorylation; P:nucleotide catabolic process
Csp_P_1969	aminopeptidase	0.0E0	45	F:aminopeptidase activity; P:proteolysis
Csp_P_1970	peptidase m4	0.0E0	53	F:metalloendopeptidase activity; P:proteolysis
Csp_P_2192	phopq-activated pathogenicity-related family protein	0.0E0	53	-
Csp_P_2420	membrane protein	0.0E0	51	C:mitochondrial outer membrane; P:transmembrane transport
Csp_P_2524	chitinase	0.0E0	56	C:extracellular region; F:chitinase activity; F:carbohydrate binding; P:carbohydrate metabolic process; P:chitin catabolic process; P:cell wall macromolecule catabolic process

Csp_P_2539	dna starvation stationary phase protection protein	4.9E-110	17	C:cell; F:DNA binding; F:ferric iron binding; F:oxidoreductase activity, oxidizing metal ions; P:cellular iron ion homeostasis; P:response to stress; P:oxidation-reduction process
Csp_P_2716	peptidase m66	0.0E0	85	F:metalloendopeptidase activity
Csp_P_2768	chitinase	0.0E0	85	C:extracellular region; F:chitinase activity; F:carbohydrate binding; P:carbohydrate metabolic process; P:chitin catabolic process; P:cell wall macromolecule catabolic process
Csp_P_2813	aspartate 1-decarboxylase	4.3E-86	14	C:cytoplasm; F:aspartate 1-decarboxylase activity; P:alanine biosynthetic process; P:pantothenate biosynthetic process; P:aspartate metabolic process; P:beta-alanine metabolic process
Csp_P_3126	peptidase m32	0.0E0	56	F:metallocarboxypeptidase activity; P:proteolysis
Csp_P_3312	omega amino acid--pyruvate aminotransferase	0.0E0	48	F:beta-alanine-pyruvate transaminase activity; F:pyridoxal phosphate binding; P:alanine metabolic process; P:aspartate metabolic process; P:isoleucine catabolic process; P:leucine catabolic process; P:valine catabolic process; P:beta-alanine metabolic process
Csp_P_3545	pkd domain-containing protein	0.0E0	56	F:calcium ion binding; F:molecular_function; P:biological_process
Csp_P_3662	probable porin signal peptide protein	0.0E0	40	C:membrane; F:porin activity; P:transport
Csp_P_3936	peptide abc transporter substrate-binding protein	0.0E0	58	C:ATP-binding cassette (ABC) transporter complex; P:transmembrane transport

Csp_P_4178	n-acetylglucosamine-binding protein a	0.0E0	76	F:hydrolase activity, hydrolyzing O-glycosyl compounds; F:carbohydrate binding; P:carbohydrate metabolic process; C:extracellular region
Csp_P_4231	tail protein	0.0E0	50	-
Csp_P_4370	peptidase m35	0.0E0	40	F:metalloendopeptidase activity; P:proteolysis
Csp_P_4488	TonB-dependent receptor	0.0E0	93	C:cell outer membrane; F:receptor activity; P:transport; P:signal transduction
Csp_P_4489	ligand-gated channel	0.0E0	91	C:cell outer membrane; F:receptor activity; P:transport; P:signal transduction
Csp_P_4696	succinylarginine dihydrolase	0.0E0	48	F:N-succinylarginine dihydrolase activity; P:arginine catabolic process to glutamate; P:arginine catabolic process to succinate

Mechanism of *Csp_P* derived anti-DENV activity

In previous studies we have shown a potent anti-DENV activity carried by *Csp_P* secreted proteins. The anti-DENV activity was consistently measured by plaque assays in which we pre-incubate *Csp_P* derived samples and DENV2 for 1 hour prior to infection. However, the mechanism by which the *Csp_P* secreted factors inhibit DENV replication *in vitro* was unknown. We modified the plaque assay in a way that would allow us to identify the stage at which DENV replication is inhibited by *Csp_P* factors.

- **Studies on the infection-stage specificity of the anti-DENV activity**

To characterize the infection stage specificity of the anti-DENV activity conferred by *Csp_P* secreted factors, the *Csp_P* supernatant was either added to the BHK21 cells prior to infection, or at the time of infection, or post infection, or incubated with DENV for 1 hour prior to infection. Anti-DENV activity of *Csp_P* supernatant in each sample was measured by plaque assays (Figure 12). Our results showed that pre-treatment of BHK21 cells with *Csp_P* supernatant has no effect on DENV replication, suggesting the target of *Csp_P* factors is less likely to be a host factor. Interestingly, dramatic anti-DENV activity was observed when we pre-incubate DENV with *Csp_P* supernatant for at least 1 hour prior to infection (Figure 12. pre-incubation), however, when we co-exposed cells with DENV and *Csp_P* supernatant, we didn't observe any anti-DENV activity (Figure 12. co-infection). One possible cause of this discrepancy could be the inhibition kinetics of *Csp_P* factors is affected by interaction between outer components of the virion and the cellular receptor. To clarify this

we arrested DENV infection at the attachment step and extended our analysis on the inhibitory effect of *Csp_P* factors.

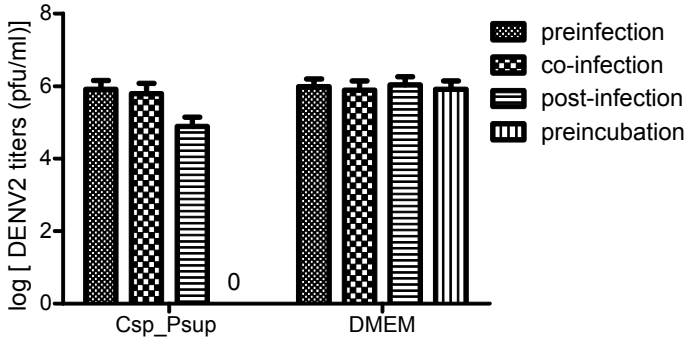

Figure 12. Time course studies on the anti-DENV activity. *Csp_P* supernatant were added to treat BHK21 cells or DENV2 in different orders. Pre-infection: BHK21 cells were treated with *Csp_P* supernatant for 1 hour, washed with PBS and infected with DENV2. Co-infection: *Csp_P* supernatant was mixed with DENV2, series diluted, and co-infect BHK21 cells for 1 hour. Post-infection: BHK21 cells were infected with DENV2 for 1 hour, washed with PBS, and treated with *Csp_P* supernatant for 1 hour. Preincubation: DENV2 was preincubated with *Csp_P* supernatant for 1 hour prior to infection. Mixture was series diluted and infect BHK21 cells for 1 hour. DENV2 titers were measured by plaque assay on the 5 th day post infection. DENV2 replication was not significantly affected by pre-infection (P = 0.8749)/co-infection (P = 0.3634)/post-infection (P = 0.4063) treatment with *Csp_P* supernatant. In contrast, DENV2 replication was dramatically inhibited by pre-incubation with *Csp_P* supernatant prior to infection, as no plaque being detected by plaque assay. Error bars represent the standard error of the mean from 2 biological replicates with technical triplicates. Statistical analysis was performed using unpaired t test to compare difference between Csp_P supernatant treatment and mock treatment with DMEM medium.

- **Virus attachment assay**

Cellular attachment of the virus particles is the first step that initiates virus entry and all the following steps necessary to unpack viral genome. Here we performed pre- and post-attachment assays while manipulating the receptor-

mediated endocytosis through a low temperature. By incubating DENV with pre-chilled cells at 4°C, we could arrest DENV infection at the attachment step and analyze the effect of *Csp_P* proteins on DENV without interference from receptor binding. In the pre-attach assay, we incubated DENV with *Csp_P* proteins for 1 hour at room temperature. After incubation, we exposed DENV-*Csp_P* proteins to pre-chilled cells at 4°C for cellular attachment. Unbound virions were washed off after 1 hour attachment. Attached virions were allowed to complete infection at 37°C. In the post-attachment assay, DENV virions were exposed to pre-chilled cells at 4°C for 1 hour attachment. After extensive wash, *Csp_P* proteins were added to treat attached-virions for 1 hour at 4°C. After another round of wash, attached virions were allowed to complete infection at 37°C. Our results showed a significant decrease ($P = 0.0002$) in DENV titers as we treated DENV with *Csp_P* proteins before viral attachment (Figure 13. Pre-attach). On the contrary, *Csp_P* proteins failed to inhibit DENV replication when attachment had already occurred (Figure 13. Post-attach). Thus, *Csp_P* proteins are most likely inhibiting the cellular attachment step of DENV.

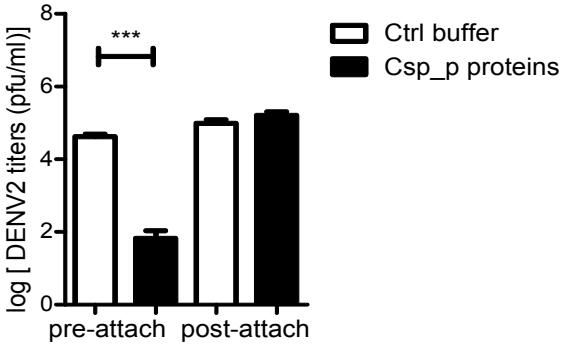

Figure 13. Attachment assay. *Csp_P* proteins/control buffer was added as treatment before or after DENV2 attachment to BHK21 cells at 4°C. After 1

hour treatment, cells were moved to 37°C in order to complete endocytosis. DENV2 titers were measured by plaque assay on the 5th day post infection. DENV2 replication was dramatically inhibited by treatment with *Csp_P* proteins prior to attachment (P = 0.0002) rather than post attachment (P = 0.2060). Error bars represent the standard error of the mean from 3 biological replicates with technical triplicates. Statistical analysis was performed using unpaired t test to compare difference between *Csp_P* proteins treatment and mock treatment with control buffer.

We next sought to validate this result by immunofluorescence microscopy. Attachment of DENV virions to a monolayer of BHK-21 cells were arrested at 4°C, fixed and stained by anti-E protein. The effect of preincubation with *Csp_P* proteins on DENV attachment was visualized under the microscope. Consistent with result from attachment assay, preincubation with *Csp_P* proteins dramatically inhibited DENV attachment to BHK21 cells, leading to decreased fluorescence intensity of membrane-bound DENV2 (Figure 14).

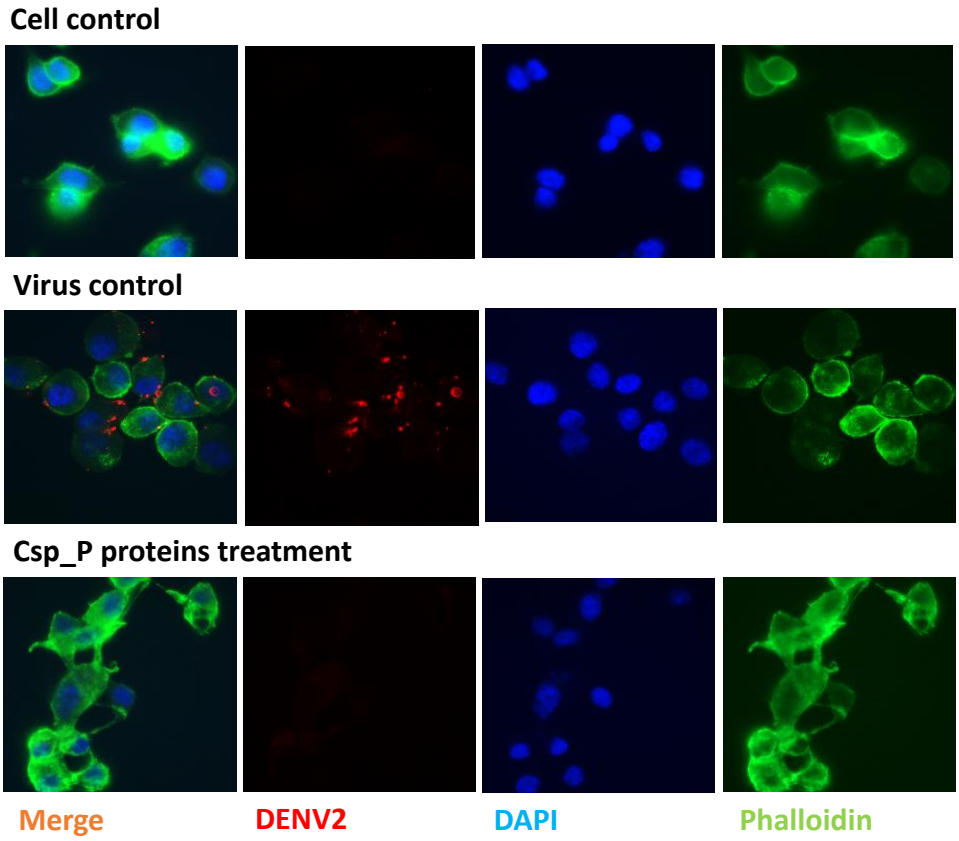

Figure 14. *Csp_P* proteins abolish DENV2 attachment to BHK21 cells. Cell control: BHK 21cells were fixed and stained with DAPI (nucleus: blue), Phalloidin (cell actin: green) and anti-DENV2 E protein (red) for microscopy. Virus control: DENV2 were added to BHK21 cells (MOI=10) for 1 hour attachment at 4°C. After twice of PBS washes, DENV2-bounded cells were fixed and stained for microscopy. *Csp_P* proteins treatment: DENV2 treated with *Csp_P* proteins for 1 hour were added to BHK21 cells (MOI=10) together for 1 hour attachment at 4°C. DENV2 with normal attachment was detected in Virus control (red), while treatment with *Csp_P* proteins effectively abolished DENV2 attachment.

- **Effect of Csp_P proteins treatment on DENV integrity**

The data described earlier showed that a 1 hour exposure of DENV to *Csp_P* proteins can effectively inhibit viral attachment of DENV. Given that the E protein of DENV essentially mediates viral attachment and entry into host cells, it raised a question whether the DENV E protein is still functional or not after treatment with *Csp_P* proteins. To address this question, DENV virions treated with *Csp_P* proteins were subjected to western blot using rabbit anti-DENV2 envelope polyclonal antibodies. As illustrated in Figure 15, treatment of DENV with *Csp_P* proteins resulted in a significant loss of the E protein, compared to the untreated DENV virions. More importantly, a cleaved fragment (about 17kDa) of DENV E protein was detected in *Csp_P* proteins treated virions (here we used a rabbit/IgG targets to a region within amino acid 1 and 495 of DENV E protein). None of *Csp_P* proteins has cross-reactivity with the used antibodies (data not shown). Based on our current results, one possible explanation for the abolished viral attachment could be enzymatic cleavage of DENV E protein that required for receptor binding.

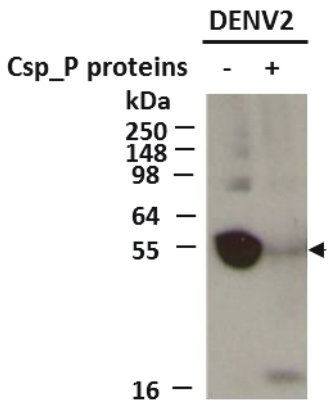

Figure 15. *Csp_P* proteins treatment leads to loss of dengue E protein. DENV2 virions were treated with *Csp_P* proteins or mock-treated with control buffer in Eppendorf tubes. After 1 hour incubation at room temperature, samples were clarified by 100kDa membrane filter at 5000*g* for 15mins and subjected to immunoblotting with a rabbit polyclonal antibody against dengue type 2 Envelope (E) protein. Arrow: E proteins in DENV2 virions. DENV2 treated with *Csp_P* proteins show decreased signal intensity for E proteins. In control experiments, DENV2 alone can be detected by anti-E as illustrated by arrow on the right.

Limitations of the current study

Further separation of *Csp_P* 50-100kDa protein fraction should be conducted using a combination of chromatography methods: for example by size-exclusion-chromatography followed by ion-exchange-chromatography. We tried to use a DENV affinity column to capture the putative DENV binding partner, contained in the *Csp_P* secretome, however, the immobilization of DENV virions on a stationary phase presented technical difficulties. We also failed to validate a possible association between *Csp_P* secreted proteins and DENV virions using a co-immunoprecipitation assay, presumably due to the cleavage of virion surface proteins. Additionally, most of our results were solely based on *in vitro* plaque assays, while they would have been more robust if based on more than one methodology for antiviral measurement (Teixeira et al., 2014).

Future directions

Our proteomic analysis for the identification of the *Csp_P*-derived factor(s) that mediate DENV inhibition will be a major focus of future work. Given the observed cleavage of the DENV E protein by a *Csp_P*–derived factor we will first focus on *Csp_P* proteases (mostly metalloproteases). Modern bioinformatics tools can be used to perform protein cleavage sites prediction based on the function of enzyme in interest. However, we certainly need to validate the kinetics of enzymatic reaction between the putative *Csp_P* -produced protease and DENV E protein. Nevertheless, a purified or expressed protease is required for these studies.

A fad-linked oxidase that was also contained in the *Csp_P* secreted protein extract shows sequence similarity to a cholesterol oxidase (CHOD) produced by other strains of *Chromobacterium*. CHODs are found exclusively in bacteria and are able to catalyze the oxidation and isomerization of cholesterol (Reiss, Faccio, Thöny-Meyer, & Richter, 2014). One study has shown that cholesterol depletion inactivates the Xenotropic murine leukemia virus-related virus (XMRV, an enveloped virus) and leads to viral envelope protein release from virions (Tang, George, Taylor, & Hildreth, 2012). In our case, we observed a significant decrease in the amount of the E protein on DENV virions upon *Csp_P* protein extract treatment, along with abolished infectivity. A study by Carro & Damonte, 2013 showed that preincubation of virions with a cholesterol extracting compound significantly reduced virus infectivity in a dose-dependent manner. Parallel to our results, they also demonstrated no effect on DENV infection when cells had been pre-treated with the same compound. Several studies have indicated a stringent requirement for membrane components, especially with respect to the percent of cholesterol and lipids, at various steps of the flavivirus life cycle (Acosta et al., 2014)(Mosso, Galván-Mendoza, Ludert, & del Angel, 2008) (Lee et al., 2008). For example, Umashankar et al., 2008 showed that the removal of cholesterol from mammalian cells will inhibit DENV production whereas studies with insect cells demonstrated that cholesterol is not required for DENV entry and membrane fusion. This could be explained by the intrinsic differences in membrane cholesterol-levels between different cell lines, and differences in the required cholesterol content for DENV propagation.

A purification strategy for microbial-derived CHOD has been previously reported in Yagi, 1973, and more recently by Doukyu, Shibata, Ogino, & Sagermann, 2008. They isolated and identified *Chromobacterium sp.* DS-1 which produces a 58kDa extracellular CHOD which has a similar molecular weight to the molecule of interest in our study. We could use a similar purification approach to isolate the CHOD from *Csp_P* proteins and characterize its anti-DENV activity.

CONCLUSIONS

We have discovered a potent *in vitro* anti-DENV activity of a *Csp_P* produced protein extract. The *Csp_P*–mediated antiviral activity appears to target the integrity of the DENV envelope protein, resulting in a compromised viral attachment to cells. While the identification of the specific anti-DENV protein is still on-going, a *Csp_P*-derived cholesterol oxidase and several proteases, identified by HPLC-MS, are likely candidates for being responsible for the observed anti-DENV activity.

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Peer-reviewed publications

Journal

Li, D., Zhang, J., Jia, X., Fang, J., Wang, M., 2014. Study on the anti-melanoma activities of fully human antibody against VEGFR-2 in vitro and in vivo, *J. Pharmaceutical Biotechnology.* 4, 311-316

Poster presentations

Fang, J., Anglero-Rodriguez, Y.I., Dong, Y., Dimopoulos G. (2016, February). Proteins derived from a mosquito midgut *Chromobacterium* isolate inhibit denue virus replication *in vitro*. 2016 Delta Omega Scientific Poster-Competition, Baltimore, MD.

Fang, J., Anglero-Rodriguez, Y.I., Saraiva R.G., Dimopoulos G. (2015, October). Protein-like factors derived from a mosquito-microbiota isolate, *Chromobacterium. Csp_P*, reduce dengue virus infection. Poster session presented at the annual meeting of the American Society of Tropical Medicine and Hygiene, Philadelphia, PA.

Leadership Experiences

- Co-founder, ZikAvoid 04/2016 – present
  Johns Hopkins CBID-Jhpiedo Emergency Zika Design Challenge, Baltimore MD
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