Role of the yellow fever virus structural protein genes in viral dissemination from the *Aedes aegypti* mosquito midgut

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Live-attenuated virus vaccines are key components in controlling arboviral diseases, but they must not disseminate in or be transmitted by mosquito vectors. Although the cycles in which many mosquito-borne viruses are transmitted are well understood, the role of viral genetics in these processes has not been fully elucidated. *Yellow fever virus* (YFV) is an important arbovirus and the prototype member of the family *Flaviviridae*. Here, YFV was used in *Aedes aegypti* mosquitoes as a model to investigate the genetic basis of infection and dissemination in mosquitoes. Viruses derived from infectious clones and chimeric viruses with defined sequential manipulations were used to investigate the influence of specific sequences within the membrane and envelope structural protein genes on dissemination of virus from the mosquito midgut. Substitution of domain III of the envelope protein from a midgut-restricted YFV into a wild-type YFV resulted in a marked decrease in virus dissemination, suggesting an important role for domain III in this process. However, synergism between elements within the flavivirus structural and non-structural protein genes may be necessary for efficient virus escape from the mosquito midgut.

INTRODUCTION

Mosquito-borne flaviviruses such as *Yellow fever virus* (YFV), *Dengue virus* (DENV) (serotypes 1–4), *Japanese encephalitis virus* (JEV) and *West Nile virus* (WNV) cause millions of infections and tens of thousands of deaths each year (Petersen & Marfin, 2005; WHO, 2003). The lack of effective antiviral measures and the failure of mosquito-control programmes in many affected areas make vaccination the primary means of prevention and, in the case of YFV, of control of human outbreaks of these diseases. Considerable effort is being directed towards the development of live virus vaccines against DENV 1–4 and WNV and an improved vaccine for JEV (Hombach et al., 2005; Monath et al., 2005). It is imperative that live-attenuated vaccine viruses should not replicate in, disseminate in or be transmitted by mosquito vectors. Our goal in these studies was to identify flavivirus genes or partial genes that, when modified appropriately, would block dissemination and transmission of virus by mosquitoes. These regions can then be targeted in the rational design of future live flavivirus vaccine candidates.

Little is known about the viral genetic factors that mediate the processes of infection, replication and dissemination in mosquito vectors, despite approximately a century of study on the ecology and epidemiology of mosquito-borne viruses. A number of steps must be completed successfully for transmission of a mosquito-borne virus to occur. Following uptake of an infectious blood meal by a female mosquito, virus must infect the midgut epithelium and replicate. To develop a disseminated infection, virus must then escape the midgut into the haemocoel and infect secondary tissues such as the salivary glands. This step is critical in allowing transmission of virus from a mosquito to a susceptible host. Transmission may then occur through deposition of virus-containing saliva when the mosquito feeds on a new host. These processes are governed by viral, vector and environmental factors (Higgs, 2004). Here, we investigated the viral genetic factors that had an effect on mosquito-vector competence, using YFV in *Aedes aegypti* as a model system. The model was based on phenotypic differences between the prototype wild-type Asibi strain and the attenuated 17D vaccine strain derived by multiple passages of the Asibi strain in tissue culture. Whereas the Asibi strain of YFV is able to infect and disseminate in a high proportion of *Ae. aegypti* individuals, 17D can infect the mosquito midgut, but does not disseminate to other tissues such as the mosquito salivary glands and so fails to be transmitted (Jennings et al., 1994; Miller & Adkins, 1988; Whitman, 1939).

The genome of YFV is 10862 nt and encodes three structural and seven non-structural proteins (3411 aa), which are flanked at the 5’ and 3’ ends by non-coding regions (NCRs).
For all flaviviruses, the envelope (E) protein is the major surface protein that mediates binding of virions to target surfaces and fusion with the membrane. The E protein has been divided into three structural domains based on sequence alignments and crystallographic structures of the E proteins of DENV and *Tick-borne encephalitis virus*. Domain I (dI) forms a β-barrel, dII is the dimerization domain and dIII is an Ig-like domain believed to function in receptor binding. The membrane (M) protein and its precursor, prM, stabilize and chaperone the E protein through the secretory pathway (Linderbach & Rice, 2003; Modis et al., 2004; Op De Beeck et al., 2004; Rey et al., 1995).

This study characterized the roles of the complete structural protein genes region (M–E), the M protein gene, and the E protein domains I–II (E dI–II) and III (E dIII) with respect to infection, virus production and dissemination in orally infected *Ae. aegypti* mosquitoes. We report here studies of eight viruses, including the parental YFV Asibi and 17D strains (Fig. 1). Two full structural chimeras, Asibi/17D M–E and 17D/Asibi M–E, were constructed and tested and the structural gene region was then further subdivided. Three chimeras, Asibi/17D E dI–II, Asibi/17D E dIII and 17D/Asibi E dIII, were produced to evaluate the roles of the E protein structural domains, with emphasis on the putative receptor-binding dIII. A Leu→Phe substitution at position 36 in the M protein was also analysed individually (Asibi/17D M–36) as it is one of two substitutions, the only in the structural protein genes, in common with another YFV strain, French neurotropic vaccine (FNV), which is not transmitted by mosquitoes (Wang et al., 1995).

**METHODS and viruses.** BHK (baby hamster kidney), Vero (African green monkey kidney) and C6/36 (*Aedes albopictus*) cells were grown in Leibovitz L-15 medium supplemented with 10% fetal bovine serum, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. BHK and Vero cells were maintained at 37°C and C6/36 cells were maintained at 28°C. An Asibi isolate of YFV was obtained from the World Reference Center for Arboviruses at the University of Texas Medical Branch (UTMB), Galveston, TX, USA. Stock virus was produced in C6/36 cells as described previously (McElroy et al., 2005). The Asibi sequence has been deposited in GenBank under accession no. AY640589.

**Construction of YFV variant infectious clones.** Construction and characterization of the Asibi infectious clone (IC) has been described previously (McElroy et al., 2005). The Asibi IC and pACNR/FLYF 17D, the 17D IC (Bredenbeek et al., 2003), were used as the backbones for all constructs. Sequence comparisons of the Asibi and 17D ICs used in this study were made by using GENERUNNER. Each YFV variant IC was constructed by using PCR-based mutagenesis and restriction digestion, followed by the exchange of DNA fragments between the Asibi and 17D ICs. RT-PCR was performed by using random hexanucleotide primers (Promega), Superscript II (Invitrogen) and *Pfu* DNA polymerase (Stratagene) under standard cycling conditions. Restriction digestion of PCR products at unique sites in the full-length Asibi and 17D ICs followed the manufacturer’s protocols (New England Biolabs), with the addition of calf intestinal phosphatase to digestion reactions containing the full-length Asibi or 17D IC as vectors for the insertion of DNA fragments. Fragments for all experiments were ligated with T4 DNA ligase (Invitrogen) and amplified in *Escherichia coli* MC1061 competent cells. To facilitate cloning during the construction of the Asibi IC, site-directed mutagenesis was used to engineer a unique BspEI restriction site at nt 494 in pACNR/FLYF 17D to produce the 17D-C494 IC. The Asibi/17D M–E IC was produced following restriction digestion of the 17D-C494 IC at the BspEI and *Mlu*I sites, gel purification of the resulting 17D DNA fragment and cloning of this fragment into the BspEI and *Mlu*I sites in the Asibi IC. The 17D/Asibi M–E IC was constructed similarly by insertion of the Asibi BspEI–*Mlu*I fragment into the 17D-C494 IC. The Asibi/17D M–36 IC was engineered by using fusion PCR of two overlapping Asibi fragments to introduce a C→T mutation at Asibi nt 854 and the PCR product was cloned into the unique BspEI and *Apa*I restriction sites in the Asibi IC. Fusion PCR and restriction digestion were also used to construct the Asibi/17D E dI–II IC, resulting in the insertion of a 1112 bp 17D DNA fragment (nt 493–1604) into the Asibi IC. Simultaneous ligation and cloning of three DNA fragments were employed to assemble the Asibi/17D E dIII and 17D/Asibi E dIII ICs. For the Asibi/17D E dIII IC, a 640 bp 17D PCR product (nt 1522–2161) was digested with *Apa*I and a 810 bp Asibi PCR product (nt 2162–2971) with *Mlu*I. Both fragments were phosphorylated with polynucleotide kinase (New England Biolabs) and

![Fig. 1. Schematic representation of YFV parental and chimeric viruses used in this study. Shading represents the sequence source. Shared sequences are those regions that do not differ between Asibi and 17D.](image-url)
cloned into the Apal and MluI sites in the Asibi IC. The 17D/Asibi E dIII IC was constructed following the same protocol, but Apal-digested Asibi and MluI-digested 17D PCR products were cloned into the Apal and MluI sites in the 17D-C949 IC. Following PCR amplification of the Asibi or 17D fragments, direct sequencing was performed for each IC at the UTMB Protein Chemistry Laboratory to confirm the introduction of genetic changes. Maps of all viruses used in this study are available from the authors on request.

**Viruses production and specific infectivity assay.** Virus was produced from cDNA ICs as described previously (McElroy et al., 2005). Briefly, RNA was produced by *in vitro* transcription of each linearized IC using an SP6 mMessage mMachine Capped RNA Transcription kit (Ambion). RNA was then purified by phenol/chloroform extraction, ethanol-precipitated and electroporated into BHK cells using the Gene Pulser Xcell electroporation system (Bio-Rad). Virus and cells were incubated in 25 cm² flasks until 75 % cytopathic effect was observed (2–4 days post-electroporation), at which time virus was aliquotted and stored at −80 °C for later use or presented to *Ae. aegypti* mosquitoes in an artificial blood meal. One aliquot of each virus was used for sequencing over the mutated region to confirm that engineered mutations were retained following virus production. We measured the specific infectivity of RNA produced from each YFV IC as described previously (McElroy et al., 2005; Yun et al., 2003). Briefly, electroporated cells were diluted ten-fold into Vero cells in suspension and a semi-solid overlay of 0-6 % tragacanth/2 × 1-15 was applied after the cells had formed a monolayer. At 4 days post-infection (p.i.), the overlay was removed and cells were washed with PBS, dried and fixed with 1 : 1 methanol : acetone. Cells were stained by immunohistochemistry using a YFV-reactive polyclonal antibody, MA93, diluted 1:500 in 1 % normal horse serum (NHS) in PBS. Signal amplification was achieved by using a peroxidase-conjugated anti-mouse IgG diluted 1:500 in 1 % NHS/PBS and a VIP Peroxidase Substrate kit (Vector Laboratories). Foci were visualized, counted and measured (10 foci per virus) by using an Olympus IX-71 inverted light microscope. Differences in focus size were tested for significance with a two-tailed *t*-test using SPSS version 11.5 (SPSS Inc.).

**Oral infection of mosquitoes.** The laboratory-adapted RexD strain of *Ae. aegypti* was used for all experiments. This strain was chosen because it has a known high susceptibility to YFV infection (Miller & Mitchell, 1991). Virus harvested fresh from electroporated BHK cells was mixed with an equal volume of defibrinated sheep blood and 3 mM ATP to make the artificial blood meal. Freshly harvested virus and cells were incubated in 25 cm² flasks until they were analysed for infection by titration on Vero cells. Infection was reported as the number of positive mosquitoes per total number of mosquitoes tested and dissemination was reported as the number of mosquitoes with YFV antigen-positive salivary glands per total number of infected mosquitoes. Differences in infection and dissemination rates were tested for significance with Fisher’s exact test using SPSS version 11.5.

**RESULTS**

**Sequence comparison of Asibi and 17D strains**

Sequence comparison of the Asibi and 17D strains used in this study revealed 67 nucleotide differences, encoding 33 amino acid substitutions scattered throughout the genome with the exception of the 5’ NCR and the capsid (structural) protein gene, which were not different. Within the remaining structural protein genes, we detected one amino acid difference in the M protein gene at position 36 and 12 amino acid differences in the E protein gene (Table 1): two amino acid differences, at aa 170 and 173, were detected in dII; three amino acid differences, at aa 52, 56 and 200, were detected in dII; five differences, at positions aa 299, 305, 325, 331 and 380, were detected in dIII; and two differences, at aa 407 and 416, were detected at the carboxyterminal end of the E protein.

**Virus production**

Following *in vitro* transcription of linearized cDNA infectious clones containing Asibi and/or 17D genetic sequences and electroporation of RNA into BHK cells, we recovered infectious virus from all clones under investigation. Introduced genetic changes following virus production in BHK cells were maintained, as confirmed by direct sequencing for each virus tested. The specific infectivity of each of the viral RNAs was 1·7 × 10⁹–3·3 × 10¹⁰ focus-forming units (µg RNA)⁻¹ and foci sizes varied for the different viruses (Table 2). As expected, 17D produced into the haemocoeel by using glass needles fashioned from pulled capillary tubes. Mosquitoes were incubated at 28 °C with 80 % humidity for up to 10 days and provided with sugar *ad libitum*.

**Evaluation of infection and dissemination.** All manipulations of live, infected mosquitoes were carried out in a Biosafety Level 3 Insectary Facility located at the UTMB. Virus production in orally infected mosquitoes was assayed by removing mosquitoes from the cages at 0, 1, 2, 3, 7 and 14 days p.i. Individual mosquitoes were titrated separately by tenfold serial dilution in Vero cells as described previously (Higgs et al., 1997; McElroy et al., 2005; Vanlandingham et al., 2005), and titres were reported as log₁₀ TCID₅₀ per mosquito. Differences in titre were analysed statistically with a two-tailed *t*-test using SPSS version 11.5. The remaining mosquitoes were incapacitated by chilling at 14 days p.i. (for oral infection experiments) or 10 days p.i. (for intrathoracic inoculation experiments), removed from the cage and the salivary glands were dissected from each body. The salivary glands were transferred to glass slides, dried and fixed in cold acetone for 10 min. Carcasses were stored at −80 °C until they were analysed for infection by titration on Vero cells. Dissected salivary glands and titration plates were stained for YFV antigen by indirect immunofluorescence assay using YFV-reactive polyclonal antibody MA93, as described previously (McElroy et al., 2005), and samples were examined under UV light with a fluorescein filter using an Olympus IX-70 epifluorescence microscope. Infection was reported as the number of positive mosquitoes per total number of mosquitoes tested and dissemination was reported as the number of mosquitoes with YFV antigen-positive salivary glands per total number of infected mosquitoes. Differences in infection and dissemination rates were tested for significance with Fisher’s exact test using SPSS version 11.5.
larger foci (0·380 mm) than did Asibi (0·191 mm; P<0·01); this was in agreement with relative plaque sizes reported previously for the two viruses (Miller & Adkins, 1988). Foci formed from each of the YFV chimeras were also significantly larger than those formed from Asibi (P<0·01). All YFV chimeras, with the exception of Asibi/17D M-36, had significantly smaller foci than did 17D (P<0·05); Asibi/17D M-36 formed larger foci than did 17D (P<0·01). Among all of the YFV chimeras, only foci formed from Asibi/17D M–E and Asibi/17D E dI–II were not statistically significantly different in size (P=0·75). We hypothesize that the larger focus size for 17D is probably due to its adaptation for growth in tissue culture as part of the vaccine-development process, rather than a reflection of virus attenuation in vivo. Previous studies of DENV (Blaney et al., 2002) and YFV (Dunster et al., 1999) have correlated plaque size with combined mutations in the structural protein genes, non-structural protein genes and NCRs. In the case of the YFV chimeras, interaction between specific sequence elements in the structural and non-structural protein genes of Asibi and 17D may account for the observed differences in the size of the foci.

Intrathoracic inoculation of Ae. aegypti

Ae. aegypti mosquitoes were inoculated intrathoracically, bypassing the midgut, with Asibi, 17D or 17D/Asibi M–E and maintained for 10 days. Surviving mosquitoes were dissected and analysed for titre, infection and dissemination (Table 3). Equivalent virus titres were recorded for each group at 10 days post-inoculation. As expected, 100% of mosquitoes examined for each virus were infected at 10 days post-inoculation. Additionally, 100% of mosquitoes tested in each of the three groups had antigen-positive head tissue and salivary glands, indicating that all three viruses were equally efficient at infecting secondary tissues within the mosquito once the midgut was bypassed.

Infection and dissemination of parental and chimeric YFV in orally infected Ae. aegypti

The viral genetic basis of infection and dissemination in mosquitoes was evaluated by infecting female Ae. aegypti mosquitoes orally with each of the viruses and maintaining them for 14 days. We characterized the remaining mosquitoes at 14 days p.i. for infection by viral titration and evaluated dissemination by staining dissected salivary glands for viral antigen (Table 4), as described previously (Higgs et al., 1997; McElroy et al., 2005). All viruses were orally infectious to Ae. aegypti. The ability to infect mosquitoes varied among the different viruses, but there was no apparent correlation with the mean day 0 blood meal titre determined for each virus. Asibi and 17D displayed the expected phenotypes in Ae. aegypti; whereas Asibi infected a high proportion of mosquitoes (72%) and disseminated in a

### Table 1. Genetic differences within the M and E protein genes of Asibi and 17D

<table>
<thead>
<tr>
<th>Protein</th>
<th>Nucleotide</th>
<th>Amino acid</th>
<th>Amino acid change (Asibi → 17D)</th>
<th>Envelope domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>854</td>
<td>36</td>
<td>Leu→Phe*</td>
<td>NA</td>
</tr>
<tr>
<td>E</td>
<td>1127</td>
<td>52</td>
<td>Gly→Arg*</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>1140</td>
<td>56</td>
<td>Ala→Val</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>1452</td>
<td>170</td>
<td>Ala→Val</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>1491</td>
<td>173</td>
<td>Thr→Ile</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>1572</td>
<td>200</td>
<td>Lys→Thr*</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>1870</td>
<td>299</td>
<td>Met→His</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>1887</td>
<td>305</td>
<td>Ser→Phe*</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>1946</td>
<td>325</td>
<td>Pro→Ser*</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>1985</td>
<td>331</td>
<td>Lys→Arg</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>2112</td>
<td>380</td>
<td>Thr→Arg*</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>2193</td>
<td>407</td>
<td>Ala→Val</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>2219</td>
<td>416</td>
<td>Ala→Thr*</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Non-conservative amino acid changes.
NA, Not applicable.

### Table 2. Specific infectivity and focus sizes of YFV variants

For clarity, statistical differences in mean focus sizes between the viruses are noted in the text.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Specific infectivity*</th>
<th>Mean focus size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asibi</td>
<td>1·6×10³</td>
<td>0·191±0·01</td>
</tr>
<tr>
<td>Asibi/17D M–E</td>
<td>4·7×10³</td>
<td>0·294±0·02</td>
</tr>
<tr>
<td>Asibi/17D M–36</td>
<td>6·7×10³</td>
<td>0·406±0·01</td>
</tr>
<tr>
<td>Asibi/17D E dI–II</td>
<td>5·7×10³</td>
<td>0·292±0·02</td>
</tr>
<tr>
<td>Asibi/17D E dIII</td>
<td>1·1×10³</td>
<td>0·224±0·01</td>
</tr>
<tr>
<td>17D/Asibi E dIII</td>
<td>1·1×10³</td>
<td>0·362±0·02</td>
</tr>
<tr>
<td>17D/Asibi M–E</td>
<td>1·3×10³</td>
<td>0·274±0·01</td>
</tr>
<tr>
<td>17D</td>
<td>3·3×10³</td>
<td>0·380±0·02</td>
</tr>
</tbody>
</table>

*Focus-forming units (μg RNA)⁻¹.
Table 3. Titre, infection and dissemination rates of selected YFV variants in intrathoracically inoculated *Ae. aegypti*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean day 10 titre*</th>
<th>No. infected (%)</th>
<th>No. disseminated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asibi</td>
<td>4.73 ± 0.30</td>
<td>13/13 (100)</td>
<td>13/13 (100)</td>
</tr>
<tr>
<td>17D</td>
<td>4.73 ± 0.30</td>
<td>15/15 (100)</td>
<td>15/15 (100)</td>
</tr>
<tr>
<td>17D/Asibi M–E</td>
<td>4.95 ± 0.00</td>
<td>15/15 (100)</td>
<td>15/15 (100)</td>
</tr>
</tbody>
</table>

*Log_{10} TCID_{50} per mosquito.

high proportion of infected mosquitoes (83%) (McElroy *et al.*, 2005), 17D had a lower infection rate (30%) compared with Asibi and was unable to disseminate. All YFV variants had significantly lower infection rates than Asibi (*P*<0.01), ranging from 16 to 38%. We believe that the low infection rate in all variants, including those predominantly encoding Asibi sequences, is probably a general attenuating effect of virus chimerization, as has been observed with other flavivirus chimeras (Blaney *et al.*, 2004; Whitehead *et al.*, 2003). There was no correlation between the source of the structural protein genes (Asibi or 17D) and the corresponding infection rate, so the specific flavivirus infection determinants could not be identified definitively in these studies.

We noted significant differences in dissemination rates (*P*<0.01) between Asibi and 17D, Asibi/17D M–E, Asibi/17D E dIII and 17D/Asibi E dIII. There was no significant difference between Asibi and 17D/Asibi M–E or Asibi and Asibi/17D E dI–II in the ability of these viruses to disseminate in infected mosquitoes, but significance was noted between these variants and 17D with respect to dissemination (*P*<0.01; Table 3). In the case of 17D/Asibi M–E, the high dissemination rate recorded for this virus (69 vs 83% for Asibi; not significantly different) might indicate that one or more elements within the M and E structural protein genes are necessary to mediate this process. When this region was examined more closely, we found that substitution of dI and dII of the E protein of Asibi with those of 17D (Asibi/17D E dI–II) had no significant effect on dissemination (67%); thus, these domains of the E protein do not appear to play a critical role in allowing virus to escape from the midgut. dIII appears to be important for dissemination. Substitution of the Asibi E protein dIII with that of 17D (Asibi/17D E dIII) compromised dissemination significantly (32 vs 83% for parental Asibi; *P*<0.01). Interestingly, the same dissemination rate (31%) was noted for the virus containing the entire 17D structural region in Asibi (Asibi/17D M–E), further validating the importance of dIII in this process. Substitution of Asibi dIII in the 17D backbone (17D/Asibi E dIII) facilitated a very limited capacity for dissemination (14 vs 0% for 17D), suggesting that its role in dissemination is synergized by other Asibi sequences, possibly within the non-structural protein genes and/or the NCRs. Incorporation of the 17D M-36 substitution into Asibi virus reduced the mean dissemination rate from 83% (for the parental Asibi) to 50% and this difference was statistically significant at *P*<0.05, implying a limited role for M in the dissemination process.

Production of virus by parental and chimeric YFVs in *Ae. aegypti*

To determine whether genetic changes introduced into Asibi or 17D would affect replication of virus in the whole mosquito following oral infection, whole mosquitoes were sampled at 0, 1, 2, 3, 7 and 14 days p.i. to monitor virus production (Fig. 2). Early time points (days 0–3), representing the eclipse phase of virus production in a mosquito, were sampled to ensure that titres determined for later time points (days 7 and 14) were the result of new virus production rather than persistence of input virus. With

Table 4. Infection and dissemination rates of virus derived from YFV ICs in orally infected *Ae. aegypti*

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. mosquitos</th>
<th>No. infected (%)</th>
<th>No. disseminated of those infected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asibi</td>
<td>58</td>
<td>42/58 (72)</td>
<td>35/42 (83)</td>
</tr>
<tr>
<td>Asibi/17D M–E</td>
<td>60</td>
<td>16/60 (27)*</td>
<td>5/16 (31)*</td>
</tr>
<tr>
<td>Asibi/17D M-36</td>
<td>49</td>
<td>16/49 (33)*</td>
<td>8/16 (50)*</td>
</tr>
<tr>
<td>Asibi/17D E dI–II</td>
<td>58</td>
<td>9/58 (16)*</td>
<td>6/9 (67)</td>
</tr>
<tr>
<td>Asibi/17D E dIII</td>
<td>53</td>
<td>22/58 (38)*</td>
<td>7/22 (32)*</td>
</tr>
<tr>
<td>17D/Asibi E dIII</td>
<td>85</td>
<td>28/85 (33)*</td>
<td>4/28 (14)*</td>
</tr>
<tr>
<td>17D/Asibi M–E</td>
<td>76</td>
<td>16/76 (21)*</td>
<td>11/16 (69)</td>
</tr>
<tr>
<td>17D</td>
<td>56</td>
<td>17/56 (30)*</td>
<td>0/17 (0)*</td>
</tr>
</tbody>
</table>

*Significantly different from Asibi by using Fisher’s exact test at *P*<0.01.
†Significantly different from Asibi by using Fisher’s exact test at *P*<0.05.
respect to virus production within a mosquito, Asibi and 17D again displayed the expected phenotypes. Higher virus titres were observed in Asibi-infected mosquitoes compared with those infected with 17D (Fig. 2a), but this difference was not significant ($P > 0.05$). All viruses displayed characteristic virus production following oral infection of mosquitoes. An initial eclipse phase (days 1–3) in which infectious virus titre decreased was followed by virus production and an increase in titre in the midgut, and virus titres then remained constant or increased to day 14. We noted no significant difference in titre between Asibi or 17D and any of the YFV variants within the whole mosquito at 14 days p.i. (Fig. 2a–c).

**DISCUSSION**

Numerous studies have attempted to pinpoint the viral genes responsible for infection and dissemination in mosquitoes. Early studies of the bunyavirus La Crosse virus and the alphavirus *Venezuelan equine encephalitis virus* (VEEV) utilized monoclonal antibody-resistant variants to localize determinants of infection and/or dissemination to the G1 and E2 envelope glycoproteins, respectively (Ludwig et al., 1991; Sundin et al., 1987; Woodward et al., 1991). The VEEV E2 envelope glycoprotein was later implicated again in mosquito midgut infection by Brault et al. (2002), who constructed and tested chimeras between epizootic and enzootic VEEV strains as part of a viral adaptation study. Myles et al. (2003) found that deletion of a portion of E2 thought to be the cell receptor-binding domain of another alphavirus, *Sindbis virus*, reduced virus infectivity for *Ae. aegypti* midguts and the ability of the viruses to disseminate in the mosquitoes. Only very limited studies of the roles of the structural protein genes in mosquito infection and dissemination have been reported for flaviviruses, which are responsible for significant numbers of human infections (Petersen & Marfin, 2005; WHO, 2003). The existence of a wild-type YFV strain, Asibi, that infects *Ae. aegypti* and disseminates at a high rate, and an attenuated YFV strain, 17D, that is able to infect *Ae. aegypti* but not to disseminate (Jennings et al., 1994; Miller & Adkins, 1988; Whitman, 1939), provides an ideal model system with which to investigate the viral genetic determinants of infection and dissemination in mosquitoes.

The most significant results of this study were those for the full structural chimeras 17D/Asibi M–E and Asibi/17D M–E. The high dissemination rate of 17D/Asibi M–E was expected, given our initial hypothesis that the structural protein genes encode the determinants of virus dissemination within a mosquito. The data for this virus are interesting when compared with data published for the DENV- and WNV-ChimeriVax vaccine candidates in mosquitoes. These viruses contain the structural protein genes of wild-type viruses DENV-2 strain PUO-218 and WNV strain NY99, respectively, in the non-structural backbone of YFV 17D and were evaluated for their ability to infect and disseminate in *Ae. aegypti*, *Ae. albopictus* (DENV only) and *Culex tritaeniorhynchus* (WNV only). Neither of the chimeric viruses was able to disseminate in any of the mosquito species tested following oral infection (Johnson et al., 2002, 2003). It was unclear from those studies whether the failure of these viruses to disseminate was due to the presence of the 17D non-structural protein genes or a result of attenuation from chimerization of heterologous flaviviruses. Data presented here suggest that the latter explanation is correct, as 17D/Asibi M–E was able to disseminate in a high proportion of infected mosquitoes in our study. Given the hypothesis stated above that the structural protein genes mediate viral dissemination in mosquito vectors, the ability of Asibi/17D M–E to disseminate (31%) was interesting, as this chimera contains the structural protein genes of a non-disseminating virus in...
the non-structural backbone of a disseminating virus. Thus, chimerization of structural protein genes from an attenuated virus and non-structural protein genes of a wild-type virus does not fully attenuate virus for dissemination. This result implies that some dissemination determinants are localized within the non-structural protein genes or the 3’ NCR. Further studies will examine the roles of these genes in viral dissemination from the mosquito midgut.

Within the E protein, the identical dissemination rates of Asibi/17D M–E and Asibi/17D E dIII (31 and 32%, respectively), coupled with the finding of a high dissemination rate for Asibi/17D E dI–II (67%), indicated that dIII contains the primary determinants of dissemination within the structural protein genes. As the hypothesized flavivirus cell receptor-binding domain, this region of the E protein is probably involved in binding to an as-yet-unknown receptor or tissue to mediate viral escape from the midgut. The low dissemination rate observed for 17D/Asibi E dIII was not expected, given the conclusion that E protein dIII is the primary determinant of dissemination within the structural protein genes. However, we have found by provisional testing of other YFV chimeras that, while the substitution of 17D sequences into Asibi had an attenuating effect on Asibi, the addition of a limited portion of Asibi sequences into 17D did not restore the wild-type phenotype (K. L. McElroy, unpublished data). It is for this reason that we did not construct and test an additional chimera, 17D/Asibi E dI–II. As stated above, the high dissemination rate of Asibi/17D E dI–II indicates that this region alone does not play a significant role in dissemination. Thus, the substitution of Asibi E dI–II into 17D will probably not increase dissemination of this virus significantly compared with 17D, as we observed with 17D/Asibi E dIII. The inability of a limited portion of Asibi sequences added to 17D to restore the wild-type phenotype supports one of our general conclusions that determinants of viral phenotype are probably distributed throughout the viral genome and some possibly localize within the non-structural protein genes.

The M protein Leu36→Phe substitution is the only difference within the structural protein genes shared by YFV wild-type/attenuated virus pairs Asibi and 17D, and the wild-type French viscerotropic virus strain of YFV and its vaccine derivative, FNV (Wang et al., 1995). The other common substitution, NS4B Ile35→Met, is being evaluated in additional studies to characterize the non-structural proteins and 3’ NCR. The M protein Leu36→Phe substitution was recently found to mediate apoptosis of DENV- and YFV-infected cells (Catteau et al., 2003), which might explain its role in the attenuation of vertebrate virulence observed for the YFV 17D and FNV strains compared with their wild-type parental viruses. As FNV, like 17D, is unable to disseminate in mosquitoes (Wang et al., 1995), we hypothesize that the M protein aa 36 substitution might be partially responsible for the inability of both 17D and FNV strains to escape from the mosquito midgut. When compared with Asibi, the dissemination rate of Asibi/17D M-36 was significant (P<0.05), but was not as low as the YFV variants containing the full M–E region of 17D or containing E dIII only (significantly different from Asibi, P<0.01). Functionally, the M protein might influence the process of dissemination, most likely via an effect on folding of the E protein and particle assembly (Op De Beeck et al., 2004).

Although previous studies of other virus strains have focused on the role of the viral E protein gene in mosquito midgut infectivity and, in some cases, midgut escape (Braught et al., 2002, 2004; Myles et al., 2003; Sundin et al., 1987; Woodward et al., 1991), our data suggest that, while dIII of the flavivirus E protein and, to a limited extent, the flavivirus M protein are important, some determinants of dissemination lie outside the structural protein genes, in those genes encoding the non-structural proteins or the 3’ NCR. The mechanism by which the non-structural protein genes or the 3’ NCR may influence dissemination is unknown. For flaviviruses, the seven non-structural protein genes and the 3’ NCR function in replication, translation and virion assembly and release (Lindenbach & Rice, 2003). Based on data presented here and for the DENV- and WNV-ChimeriVax vaccine candidates, a complex set of interactions between the structural and non-structural proteins of the same virus mediates dissemination at a high rate in infected mosquitoes.

In our studies, there was no definitive indication of the viral genetic sequences within the structural protein genes that mediate infection of the midgut epithelium, as we found no apparent correlation between viral sequence within this region and infection rate. Further work will focus on sequence differences localized within the non-structural protein genes and the 3’ NCR, which may enhance or attenuate mosquito infection and/or dissemination. With respect to midgut escape/dissemination, our model revealed an association with the sequence of the structural protein genes. The mosquito midgut forms the first barrier to transmission of virus, through an infection and/or an escape barrier (Higgs, 2004). In the case of 17D, we know that failure of virus to infect the salivary glands is due to a barrier at the level of midgut escape, because when 17D is inoculated intrathoracically into Ae. aegypti (bypassing the midgut), virus replicates and infects the head tissues and salivary glands at the same rate as Asibi. The same result was recorded for 17D/Asibi M–E following intrathoracic inoculation. Thus, any YFV variant that is able to escape from the midgut would probably replicate and infect the salivary glands. Differences in dissemination rates seen among the YFV variants are thus due to the inability of virus to escape from the midgut, rather than failure to infect secondary tissues.

It is still unknown how the virus travels from an infected midgut epithelial cell through the basal lamina into the haemocoeol to infect secondary tissues such as the salivary glands. Pore sizes of the basal lamina underlying the midgut
epithelium are considerably smaller than mosquito-borne viruses, making direct egress of virions through the basal lamina to the haemocoele unlikely (Reddy & Locke, 1990). Romoser et al. (2004) hypothesized that, following infection of the midgut epithelium, virus may spread from cell to cell and virions may exit the midgut through modified porous basal lamina or by infecting midgut muscle cells or tracheal cells to escape into the haemocoele. We hypothesize that sequences within both the virus structural and non-structural protein genes and the 3’ NCR would be involved in virus dissemination based on the results of this study, as these events would be both receptor-mediated (encoded by the structural protein genes) and dependent on efficient virus replication and virion packaging and release (encoded by the non-structural protein genes and the 3’ NCR).

Elucidating the determinants of viral dissemination from the mosquito midgut not only will aid in the development of non-transmissible live virus vaccines, but will contribute to our basic understanding of mosquito biology and vector–pathogen interactions. Our focus has been the viral genetic factors driving dissemination from the midgut and much attention has been devoted elsewhere to the role of vector genetics in pathogen transmission (Holt et al., 2002; Tabachnick et al., 1985). Obtaining such knowledge is essential to the creation and implementation of effective control measures against vector-borne diseases and is thus among several goals for the field of medical entomology in the future (Tabachnick, 2003).

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