tween the Pvu I and Bgl II sites, which may account for the weak intrastrand hybridization signals seen in lane 2 of Fig. 3B or lanes 1 and 3 of Fig. 3C.

29. The DNA samples were denatured and loaded in triplicate on a 5% sequencing gel (*30*). DNA was transferred to nylon filter by electroblotting after equilibration of the gel in electroblot buffer (10 m*M* tris, 5 m*M* sodium acetate, and 0.5 m*M* EDTA, *p*H 7.8) (Bio-Rad Trans-blot instruction manual) for 30 minutes. To prepare ^{32}P-labeled single-stranded DNA probes, pNL25 plasmid DNA was digested with Bgl II and subsequently with alkaline phosphatase. To label the 5′ end of the bottom strand (B probe), we incubated the DNA with [γ-^{32}P]dATP and T4 kinase. To label the 3′ end of the top strand (T probe), we filled in the DNA with [α-^{32}P]dATP by using the Klenow fragment of *E. coli* DNA polymerase (*27*). After the labeling period, the samples were digested with Bgl I and the fragments corresponding to T or B probes were isolated from 1.2% low-melting agarose gel. After phenolization and precipitation the specific activity of the end-labeled probes was 1×10^6 to 5×10^6 cpm/μg. The B probe was used in lanes 1, 2, and 3, and the T probe in lanes 4, 5, and 6. The entire pNL25 plasmid DNA was used to make the nick-translated probe (homologous to both B and T) for hybridization shown in lanes 7, 8, and 9. Probes were boiled before hybridization, and hybridization was carried out at 65°C for 16 hours. Filters were washed with 4× SSC (1× SSC is 0.15*M* NaCl and 0.015*M* sodium citrate) and 0.1% sodium dodecyl sulfate for 30 minutes at 60°C by changing the washing solution every 10 minutes.

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31. We thank J. Gielen, R. Villarroel, A. Lenaerts, and J. Coppieters for sequence synthesis and analysis; R. Deblaere for providing the pVS1 derivative; P. Dhaese and L. Herrera-Estrella for critical reading of the manuscript; and M. De Cock, S. Elia, S. Van Gijsegem, and K. Spruyt for preparation of this manuscript and figures. Supported by a Ph.D. fellowship of the Rockefeller Foundation (to K.W.), by grants from the "A.S.L.K.-Kankerfonds," by grant 3.001.82 from "Fonds voor Geneeskundig Wetenschappelijk Onderzoek," and by grant O.O.A. 12.0561.84 of the Services of the Prime Minister (to M.V.M. and J. Schell).

11 August 1986; accepted 11 December 1986

A G1 Glycoprotein Epitope of La Crosse Virus: A Determinant of Infection of *Aedes triseriatus*

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Arthropod-borne viruses (arboviruses) have specific vector-vertebrate host cycles in nature. The molecular basis of restriction of virus replication to a very limited number of vector species is unknown, but the present study suggests that viral attachment proteins are important determinants of vector-virus interactions. The principal vector of La Crosse (LAC) virus is the mosquito *Aedes triseriatus*, and LAC virus efficiently infects the mosquito when ingested. However, a variant (V22) of LAC virus, which was selected by growing the virus in the presence of a monoclonal antibody, was markedly restricted in its ability to infect *Ae. triseriatus* when it was ingested. Only 15% of the mosquitoes that ingested V22 became infected and 5% of these developed disseminated infections. In contrast, 89% of the mosquitoes that ingested LAC became infected and 74% developed disseminated infections. When V22 was passed three times in mosquitoes by feeding, a revertant virus, V22M3, was obtained that infected 85% of *Ae. triseriatus* ingesting this virus. In addition, V22M3 regained the antigenic phenotype and fusion capability of the parent LAC virus. These results suggest that the specificity of LAC virus-vector interactions is markedly influenced by the efficiency of the fusion function of the G1 envelope glycoprotein operating at the midgut level in the arthropod vector.

ARTHROPOD-BORNE VIRUSES ARE typically restricted in their range of vector species. This vector specificity, which is a major feature of arbovirus cycles in nature, is dictated in part by the biological and behavioral attributes of the vector population. The interaction of the virus and the vector at the midgut level is another major determinant of vector specificity. When the virus has been ingested in a blood meal, it must first infect the vector's midgut epithelial cells. Progeny virus must then disseminate from midgut cells into the hemocoel, to infect the salivary glands if the virus is to be transmitted to further vertebrate hosts or to infect the ovaries if the virus is to be transovarially transmitted to further vectors (*1*). In nonpermissive vector species, arboviruses frequently are incapable of infecting the midgut cells (*1*). The molecular determinants of this midgut barrier are unknown.

One of the more attractive hypotheses to explain the midgut barrier is the presence of specific virus receptors on vector midgut cells. Virion surface proteins may act as ligands and bind to cellular receptors and may mediate early steps in infection, such as fusion of the viral envelope with cellular membranes. There is little direct evidence to suggest that such receptors function in vivo in the vector midgut; however, Western equine encephalomyelitis virus apparently penetrates mesenteronal cells by fusion (*2*).

We have used LAC virus, the causal agent of California (La Crosse) encephalitis, to investigate the midgut barrier in *Aedes triseriatus* mosquitoes. LAC virus (Bunyaviridae) is transmitted mainly by *Ae. triseriatus*, by way of the salivary glands; chipmunks and squirrels are the principal vertebrate hosts. LAC virus can also be transmitted transovarially and venereally by *Ae. triseriatus* (*3*).

The development of monoclonal antibodies to specific epitopes on the LAC virus G1 glycoprotein has made it possible to assess the role of the major envelope protein in midgut infection of *Ae. triseriatus*. These monoclonal antibodies neutralize LAC virus and inhibit hemagglutination, suggesting that the G1 protein functions as a ligand for cellular receptors (*4*). These antibodies also inhibit the fusion function of LAC virus, which is demonstrable at *p*H 6.2 or lower (*5*).

A panel of the monoclonal antibodies was used to select from the parent LAC stock virus (a mouse brain preparation, passage 6) a corresponding series of variant viruses that were monoclonal antibody–resistant (MAR). A few of the MAR variants showed reduced fusion capability and reduced virulence for mice (*5*). One such variant, V22, showed the greatest reduction in fusion efficiency and mouse neuroinvasiveness. We investigated the ability of V22 (selected with monoclonal antibody 807-22) to infect and to replicate in the La Crosse strain of *Ae. triseriatus*, which was originally collected in La Crosse, Wisconsin, in 1980.

Mosquitoes were permitted to ingest blood meals composed of equal parts of washed human red blood cells, the virus preparation, and a mixture of 50% sucrose and fetal calf serum. The virus titer in the meal preparations ranged from 6.6 to 6.8 $\log_{10}$ $TCID_{50}$ per milliliter. After 14 days of extrinsic incubation at 24°C, mosquitoes were examined by immunofluorescence for the presence of viral antigen (*6*). Heads and abdomens were severed from mosquitoes and squashed on glass slides. After fixation in acetone, tissues were stained with mouse antibodies to LAC virus conjugated with fluorescein isothiocyanate. Detection of viral antigen in the abdominal tissues indicated that the virus infected the midgut cells, while the presence of viral antigen in head

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Table 1. A monoclonal antibody–resistant variant (V22) of LAC virus with restricted ability to infect *Ae. triseriatus*. Infection rates were determined by examining the tissues for the presence of viral antigen by immunofluorescence as described in the text.

Virus	Number of mosquitoes examined	Number positive (%)	
		Abdomen	Head
Parent LAC virus	19	89	74
Variant V22	74	15	5

Table 2. Variant V22 and parental LAC virus are equally infectious after intrathoracic inoculation of mosquitoes. Virus was titrated in cell culture and in mosquitoes and the mean titers and standard deviations are presented. $TCID_{50}$: 50% tissue culture infectious dose; MID_{50}: 50% mosquito infectious dose.

Virus	Log_{10} virus titer		$TCID_{50}/MID_{50}$
	$TCID_{50}$	MID_{50}	
Parent LAC virus	7.3 ± 0.19	8.2 ± 0.19	0.13
Variant V22	6.2 ± 0.19	7.1 ± 0.19	0.13

tissues indicated that the virus had productively infected midgut cells and had disseminated to infect secondary target organs. Differential infection and dissemination rates were detected (Table 1). Parental LAC virus infected 89% of the mosquitoes and disseminated in 74%. In contrast, V22 only infected 15% of the mosquitoes that ingested the virus and disseminated in only 5%.

To determine if V22 was capable of replication in the vector, we inoculated mosquitoes intrathoracically, thus bypassed the midgut barrier. V22 and the parent virus were equivalent in their ability to infect and to replicate in mosquitoes after intrathoracic injection. The ratios of the median tissue culture infective dose to the median mosquito infective dose ($TCID_{50}/MID_{50}$) were equivalent (Table 2). Likewise, when mosquitoes were infected intrathoracically with similar doses of the two viruses, the virus titers in the mosquitoes were equal 14 days later. The geometric mean titer per mosquito infected with the parent virus was 3.8 log_{10} $TCID_{50}$ (SE, ±0.5), while the mean titer of mosquitoes infected with V22 virus was 4.0 log_{10} $TCID_{50}$ (SE, ±0.45).

To characterize the V22 that had efficiently infected mosquitoes by the oral route, we isolated virus from mosquitoes with disseminated infections. The virus was propagated in BHK-21 cells and refed to mosquitoes. After three such passages, the resultant virus, designated V22M3, infected and disseminated as efficiently in the vector as the parent LAC virus (Table 3).

To determine the neutralization phenotype of the virus, we used a microneutralization test; virus (100 plaque-forming units) was incubated in serial twofold dilutions of the monoclonal antibody 807-22 (four replicates for each dilution) for 1 hour at room temperature. Virus-antibody suspensions were added to monolayers of BHK-21 cells in 96-well microtiter plates. Cells were examined for the presence of cytopathic effect (CPE) for 5 days, and the neutralization end point is given as the reciprocal of the dilution of antibody that resulted in CPE in >75% of the wells. V22M3 and parent LAC virus were both neutralized by monoclonal antibody 807-22; V22 was not (Table 3).

We then determined the fusion index. BHK-21 monolayers were infected with the respective virus (multiplicity of infection of 0.1), incubated for 18 hours at 35°C, treated with buffer (*p*H 6.0) for 1 minute, held at 37°C for 30 minutes, fixed, and read, as previously described (*5*). V22M3 virus showed a fusion profile similar to the parent LAC virus (Table 3). The increase in infection rate, neutralization by the monoclonal antibody 807-22, and restored fusion function all suggest that V22M3 had reverted at the specific epitope.

The specificity of vector-virus interactions is illustrated by the dramatic effect of altering a single epitope upon the mosquito infectivity of LAC virus. There are two possible explanations for the altered infectivity of variant V22: decreased efficiency of infection of midgut epithelial cells or reduced virion stability in the midgut lumen. The alteration in epitope 22 could reduce efficiency of infection of midgut cells either by altered binding to a putative cellular receptor or by an alteration in the subsequent fusion of the virion envelope with a cellular membrane. Alternatively, since the midgut lumen of the mosquito is a virucidal environment, it is possible that the reduced infectivity of V22 is due to reduced virion stability in the gut. Treatment of intact LAC virions with proteolytic enzymes will cleave the G1 glycoprotein (*5*), and differential infectivity of strains of reovirus has been associated with differences in proteolytic processing (*7*). Among these alternatives, substantial evidence for the role of the fusion function (*8*) is provided by the concordance of mosquito infectivity and fusion function in parent LAC, V22, and V22M3 viruses. However, elucidation of the precise mechanism causing the reduced infectivity awaits further investigation.

It would be most interesting to determine if MAR variants of other arboviruses, such as alphaviruses and flaviviruses, exhibit altered infection and replication capability in vectors. Such studies could lead to a better understanding of the mechanisms of vector specificity and could suggest novel control strategies for these important pathogens of humans and animals.

Table 3. Reversion in the phenotype of variant V22 after passage in mosquitoes. V22 virus was passed three times by oral administration to mosquitoes as described in the text, and the comparative abilities of the parent, V22, and V22M3 to infect mosquitoes, to be neutralized by monoclonal antibody 807-22, and to fuse BHK-21 cells were determined. The techniques used to determine these phenotypes are described in the text.

Virus	Number of mosquitoes that		Percentage infected	Neutralization titer	Fusion index (*p*H 6.0)
	Received virus	Became infected			
Parent La Crosse	19	14	74	160	≥0.8
Variant V22	47	4	5	<10	0.2
Revertant V22M3	59	50	85	320	≥0.6

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16 June 1986; accepted 16 December 1986

Redesigning Metabolic Routes: Manipulation of TOL Plasmid Pathway for Catabolism of Alkylbenzoates

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Increasing quantities of man-made organic chemicals are released each year into the biosphere. Some of these compounds are both toxic and relatively resistant to physical, chemical, or biological degradation, and they thus constitute an environmental burden of considerable magnitude. Genetic manipulation of microbial catabolic pathways offers a powerful means by which to accelerate evolution of biodegradative routes through which such compounds might be eliminated from the environment. In the experiments described here, a catabolic pathway for alkylbenzoates specified by the TOL plasmid of *Pseudomonas* was restructured to produce a pathway capable of processing a new substrate, 4-ethylbenzoate. Analysis of critical steps in the TOL pathway that prevent metabolism of 4-ethylbenzoate revealed that this compound fails to induce synthesis of the catabolic enzymes and that one of its metabolic intermediates inactivates catechol 2,3-dioxygenase (C23O), the enzyme that cleaves the aromatic ring. Consequently, the pathway was sequentially modified by recruitment of genes from mutant bacteria selected for their production of either an altered pathway operon regulator that is activated by 4-ethylbenzoate or an altered C23O that is less sensitive to metabolite inactivation. The redesigned pathway was stably expressed and enabled host bacteria to degrade 4-ethylbenzoate in addition to the normal substrates of the TOL pathway.

MOST ORGANIC COMPOUNDS ARE ultimately degraded by soil microorganisms. Bacteria, for example, rapidly mineralize many substances and play important roles in the carbon cycle and in maintaining ecosystems in balance. In recent years, large quantities of synthetic organic chemicals have been released into the biosphere. Although many of these are rapidly biodegraded, others are catabolized only slowly or incompletely, if at all. Toxic members of this latter group constitute an important source of environmental pollution. Although soil and water microorganisms have considerable evolutionary potential and, under appropriate selective conditions, evolve pathways able to degrade many synthetic compounds, such conditions do not always arise in nature and the evolution of effective pathways for certain compounds may thus be relatively slow.

Genetic engineering offers considerable promise as an approach to accelerate the evolution of desired metabolic pathways (*1*). Two general strategies can be envisaged for the experimental evolution of new catabolic activities, namely, the restructuring of an existing pathway and the assembly of a new route through the functional combination of appropriate sections of different pathways. Restructuring an existing pathway by substituting enzymes or other proteins through recruitment of new genes has been successfully used to evolve derivatives of *Pseudomonas sp.* B13 that can degrade an increased range of chlorinated aromatics (*2–4*). These earlier experiments involved recruitment of single enzymes exhibiting broad substrate specificities. We now describe the restructuring of a catabolic pathway for alkylbenzoates specified by the TOL plasmid pWWO of *Pseudomonas putida* (Fig. 1), through directed evolution of existing key pathway elements, to create a pathway able to process 4-ethylbenzoate (4EB). This process involved identifying the barriers that prevent degradation of 4EB through the TOL pathway, selecting mutants exhibiting broader substrate-effector specificities in the key barrier steps, and recruiting the mutant genes to produce derivatives of *P. putida* able to degrade 4EB and utilize it for growth.

Flasks containing M9 minimal medium (*4*) plus an alkylbenzoate as sole source of carbon and energy were inoculated with soil samples from the Geneva area and incubated at 30°C. Although organisms were readily isolated that could grow on 3-methylbenzoate (3MB), 4-methylbenzoate (4MB), and 3,4-dimethylbenzoate, none was isolated that could grow on 4EB. The resistance of 4EB to degradation could in principle be due to (i) its toxicity, (ii) its failure to be taken up by the bacteria, (iii) its failure to act as an effector of regulators of transcription of catabolic operons, and (iv) its failure to be degraded by the pathway enzymes. Since 3-ethylbenzoate is catabolized by *P. putida* carrying TOL plasmid pWWO and related bacteria (*5–7*), the first two possibilities were unlikely. That they did not account for the resistance to degradation was confirmed by examining the metabolism of 4EB by *P. putida* carrying pNM72, a TOL plasmid derivative in which the benzoate *meta*-cleavage pathway (Fig. 1) is expressed constitutively at high levels (*8*). Although *P. putida* (pNM72) did not grow on 4EB as sole carbon source, when this bacterium was provided with glucose and 4EB it grew at the expense of the sugar and accumulated 4-ethylcatechol (4EC) (identified in culture supernatants by mass spectrometry after being purified by high-pressure liquid chromatography). This result suggests that 4EB is not particularly toxic, that it enters the bacteria, and that it is metabolized by the initial enzymes of the benzoate pathway.

The reason for the block in 4EB degradation at the level of 4EC is that 4EC (or its metabolite) inactivates the subsequent enzyme of the pathway, catechol 2,3-dioxygenase (C23O), the key enzyme for cleavage of the aromatic ring (Fig. 2). Inactivation was not reversed by addition of 100 μM catechol (Fig. 2) or by dialysis of the inactivated enzyme.

Plasmid pNM185 (Table 1) is an expression vector with broad host range that contains the promoter (P_m) of the TOL plasmid *meta*-cleavage pathway operon and *xylS*, the gene for the positive regulator of this promoter (*9*). Plasmid pNM189 (Table 1), a derivative of pNM185 containing *lacZ* downstream of P_m (*9*), has been used to quantify the ability of potential substrates of the TOL *meta*-cleavage pathway to activate the *xylS* protein and thereby synthesize the catabolic enzymes (*4*). Although 3MB and 4MB activated the *xylS* protein, 4EB did not (Table 2). Thus, there are at least two obstacles to the degradation of 4EB by the TOL plasmid *meta*-cleavage pathway. To redesign the TOL pathway so that 4EB could be processed through it, it was neces-

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