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7. Frozen *M. jannaschii* cells (200 g) were resuspended in two volumes of buffer A [50 mM tris-HCl (pH 8), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 2 mM benzamidine, and 10% glycerol], sonicated, and centrifuged at 120,000*g* for 1.5 hours. The sample was dialyzed overnight against the same buffer and loaded on a DE52 DEAE-cellulose column (Whatman). A linear gradient of KCl (0 to 500 mM) was applied and the active fractions were pooled and dialyzed against 20 mM phosphate buffer (pH 6.8) containing 10 mM 2-mercaptoethanol and 10% glycerol. After dialysis the sample was applied to a P11 phosphocellulose column (Whatman), and active fractions were eluted with a linear gradient of 20 to 500 mM phosphate buffer. After dialysis in phosphate buffer the active fractions were applied to a Bio-Gel HT hydroxyapatite column (Bio-Rad) that was eluted with a linear gradient of 0.02 to 1 M phosphate buffer (pH 6.8). Active fractions were concentrated with solid polyethyleneglycol 20000 in dialysis bags, dialyzed against buffer A, and fractionated again by anion-exchange chromatography with a Uno-Q column (Bio-Rad). Active samples were then loaded on a gel filtration column (Superdex 200, Pharmacia), and fractions from this separation with CysRS activity were then loaded on a tRNA affinity column (Sepharose 4B–CNBr activated, Pharmacia) prepared according to the manufacturer's instructions, with 100 mg of total *E. coli* tRNA (Boehringer Mannheim) as coupling ligand. Samples (20 μl) from the most active fractions were then analyzed on tris-glycine native gels (10 to 20% polyacrylamide, Bio-Rad). After visualization of proteins either by Coomassie Blue or silver staining, samples were excised from the gels, eluted overnight in reaction buffer B [50 mM Hepes (pH 7), 50 mM KCl, 15 mM $MgCl_2$, 5 mM dithiothreitol (DTT)] containing 1 mM benzamidine and 10% glycerol, and tested for CysRS activity. Cys-tRNA and Pro-tRNA synthesis was assayed at 70°C in reaction buffer B in the presence of total *M. jannaschii* tRNA (1 mg/ml, prepared by standard methods) and 20 μM radioactive amino acid {[^{35}S]cysteine (1075 Ci/mmol; NEN DuPont) or [^{14}C]cysteine (303 mCi/mmol, NEN DuPont; reduced by DTT)]} or proline (103 Ci/mmo, Amersham). Samples taken at various time points were spotted on Whatman 3MM filter disks, presoaked in 10% trichloroacetic acid. The disks were washed and radioactivity was measured by liquid scintillation counting.
8. The *proS* gene was cloned from *M. jannaschii* genomic DNA by polymerase chain reaction (PCR) with the primers G**CATATG**TTGGAATTTTCAGAATGGTATTCAGATATA and **GGATCC**TTAGTAGGTTTTAGCTATTGCTATATATTTATTAC containing Nde I and Bam HI restriction sites, respectively (indicated in bold). For expression in *E. coli*, *M. jannaschii proS* was subcloned into pET 15b (Invitrogen) and used to transform the strain BL21 (DE3). The derived strain was then used for the production of His_6-ProRS, which was subsequently purified by nickel-affinity chromatography (Qiagen) followed by cation exchange chromatography with a Mono-S column (Pharmacia). After these purification steps, the His_6-ProRS was judged to be at least 99% pure by Coomassie Blue staining after SDS–polyacrylamide gel electrophoresis (PAGE).
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13. *Escherichia coli cysS* and *proS* genes from *M. thermoautotrophicum* and *M. jannaschii* were cloned into plasmid pCBS1 (*19*) to yield pCBS-Ec-cysS, pCBS-Mt-proS, and pCBS-Mj-proS. After obtaining some partial sequence from the University of Washington Genome Center (http://kandinsky.genome.washington.edu/Maripaludis/html/top.html), the *M. maripaludis proS* gene was cloned from genomic DNA by PCR. The complete gene was sequenced (GenBank accession number AF163997) and cloned into pCBS1 to generate pCBS-Mm-proS. The *E. coli* strain UQ818 [*cysS*^{ts}; K. Bohman and L. A. Isaksson, *Mol. Gen. Genet.* **176**, 53 (1979)] was transformed at 30°C with these plasmids and the resulting transformants tested for growth on Luria-Bertani agar supplemented with ampicillin (100 mg/liter), chloramphenicol (34 mg/liter), and Cys (0.5 mM) at 30° and 42°C.
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A H^+-Gated Urea Channel: The Link Between *Helicobacter pylori* Urease and Gastric Colonization

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Acidic media trigger cytoplasmic urease activity of the unique human gastric pathogen *Helicobacter pylori*. Deletion of *ureI* prevents this activation of cytoplasmic urease that is essential for bacterial acid resistance. UreI is an inner membrane protein with six transmembrane segments as shown by in vitro transcription/translation and membrane separation. Expression of UreI in *Xenopus* oocytes results in acid-stimulated urea uptake, with a pH profile similar to activation of cytoplasmic urease. Mutation of periplasmic histidine 123 abolishes stimulation. UreI-mediated transport is urea specific, passive, nonsaturable, nonelectrogenic, and temperature independent. UreI functions as a H^+-gated urea channel regulating cytoplasmic urease that is essential for gastric survival and colonization.

The Gram-negative pathogen *H. pylori* is unique in its ability to colonize the human stomach. *H. pylori* infection is acquired during childhood, persists lifelong if not eradicated, and is associated with chronic gastritis and an increased risk of peptic ulcer disease and gastric cancer (*1*). An acid-tolerant neutralophile, *H. pylori* expresses a neutral pH–optimum urease to maintain proton motive force (PMF) and to enable gastric colonization (*2*).

Most urease is found in the bacterial cytoplasm, although up to 10% appears on the surface, owing to cell lysis during culture (*3*). Surface or free urease has a pH optimum between pH 7.5 and 8.0 but is irreversibly inactivated below pH 4.0 (*4*, *5*). The activity of cytoplasmic urease is low at neutral pH but increases 10- to 20-fold as the external pH falls between 6.5 and 5.5, and its activity remains high down to pH ~2.5 (*5*). Thus, cytoplasmic, not surface, urease is required for acid resistance. The unmodified urea permeability of the inner membrane is insufficient to supply enough urea to intrabacterial urease for urease activity to buffer the bacterial periplasm in the face of gastric acidity (the median diurnal acidity of the human stomach is pH 1.4). The data here show that *H. pylori* expresses a urea transport protein with unique acid-dependent properties that activates the rate of urea entry into the cytoplasm.

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The permeability of urea across phospholipid bilayer membranes, 4×10^{-6} cm s^{-1} (*6*), is insufficient to saturate internal urease. At neutral pH, this rate of urea entry is not able to saturate intrabacterial urease even with 100 mM external urea. In acidic media, the apparent Michaelis constant K_m of internal urease becomes equal to that of free urease, ~1 mM (*5*), demonstrating an accelerated urea entry. The addition of 0.01% of the nonionic detergent $C_{12}E_8$ permeabilizes the inner membrane, as shown by penetration of propidium iodide, without disrupting its morphology (Fig. 1). The urease of $C_{12}E_8$-treated intact organisms is fully active at neutral pH (intact bacteria, 0.25 ± 0.1 μmol of urea per minute per milligram of protein; bacterial homogenate, 2.76 ± 0.27 μmol of urea per minute per milligram of protein; bacteria with 0.01% $C_{12}E_8$, 2.65 ± 0.10 μmol of urea per minute per milligram of protein). Thus, an increase of urea permeability of intact *H. pylori* accounts for the activation of cytoplasmic urease in acidic media.

The urease gene cluster consists of seven genes. *ureA* and *ureB* encode the urease structural subunits, and *ureE*, *-F*, *-G*, and *-H* encode accessory proteins necessary for Ni^{2+} insertion into the apoenzyme (*7*). *ureI* encodes a membrane protein with homology to putative amide transporters such as AmiS, AmiS2, and ORFP3 (*8*), and its absence impairs acid survival (*9*). UreI may be an acid-activated urea transporter crucial for acid resistance of *H. pylori.* Its function was determined in deletion mutants and by expression in *Xenopus* oocytes.

In contrast to the large acid-induced increase in urease activity of wild-type organisms, no increase of activity in acidic medium was observed in the intact ureI mutant (*10*), DW504UreI$^-$ (Fig. 2). However, urease activity of detergent-treated *ureI*$^-$ cells (3.0 ± 0.25 μmol of urea per minute per milligram of protein) or cell lysate resulted in urease activity equal to that of wild-type organisms. The normal level of urease shows that deletion of *ureI* did not affect the expression of the downstream urease accessory genes essential for its biosynthesis. The mutation is therefore nonpolar. Abolition of acid activation of urease in intact organisms by *ureI* deletion and full activation of intrabacterial urease by $C_{12}E_8$ suggest that UreI-mediated transport of urea determines the pH dependence of cytoplasmic urease.

Because activity of the *H. pylori* cytoplasmic urease maintains an inward urea gradient, uptake does not need energy from adenosine triphosphate (ATP) or ion gradients. Measurement of passive urea uptake in prokaryotes, with their small volume-to-surface ratio, is precluded by the endogenous permeability of phospholipid bilayers. Oocytes have a volume-to-surface ratio several hundred times that of prokaryotes and express neither endogenous urea transporters nor urease activity (*11*). UreI was therefore expressed in *Xenopus* oocytes by injection of *ureI* complementary RNA (cRNA) (Fig. 3). Over 30 min, urea uptake in UreI oocytes was accelerated 6- to 10-fold at pH 5.0 compared with pH 7.5 and was the same as in noninjected oocytes at either pH (*12*) (Fig. 4, A and C). Control oocytes equilibrated to the same level as oocytes expressing UreI, but required 48 hours to reach equilibrium as compared with 1 hour for *ureI*-injected oocytes. No increase in internal concentration was found above the increase from equalization of the concentration gradient. Accumulation was consistent with acid-dependent UreI facilitation of urea transport into the 0.4 to 0.6 μl of internal oocyte water space (*13*).

UreI-dependent urea uptake was activated with a pH profile nearly identical to the pH activation profile of cytoplasmic urease in *H. pylori* (*5*). Half-maximal activation of transport occurred at pH ~6.0 (Fig. 4B). Uptake was highly selective for urea, with only trace accumulation of ^{14}C-thiourea (Fig. 4C) or ^{14}C-mannitol (1.13 ± 0.10 and 0.41 ± 0.14 pmol per oocyte at pH 5.0, respectively). Uptake of 50 μM ^{14}C-urea was 15.28 ± 0.27 and 13.61 ± 0.97 pmol per oocyte in the absence or presence of 100 mM unlabeled urea, respectively. Thus, saturation was not seen, even though a 2000-fold excess of urea was added (Fig. 4C). The addition of urea to voltage-clamped UreI-expressing oocytes resulted in no change in current. An inward current of 117 nA is predicted, if UreI were a proton- or cation-driven urea transporter with a stoichiometry of 1:1 (*14*). UreI-mediated urea uptake is therefore nonelectrogenic.

Transport at pH 5.0 was temperature independent between 15° and 30°C (Fig. 4D). This temperature insensivity and the lack of saturation of uptake suggest that, after H^+ activation, urea fluxes through UreI with little interaction with the protein. Aquaporins, although also putative six transmembrane–segment channel-like water transport proteins, show substantial temperature dependence (*15*). Our data suggest that UreI functions as a specific, H^+-activated urea channel. A channel mechanism would allow a rate of urea uptake adequate for saturation of internal urease at physiological gastric urea concentrations (1 to 3 mM).

Western blot analysis detected the presence of UreI in purified inner but not outer membrane fractions (Fig. 3). Periodic acid–silver staining detected carbohydrate (*16*) in the outer but not inner membrane fraction (*17*), confirming the validity of the separation (*18*). UreI contains six hydrophobic sequences, H1 to H6. In vitro transcription/translation of various NH_2-terminal lengths of UreI, fused to a glycosylatable COOH-terminal tag, was used to

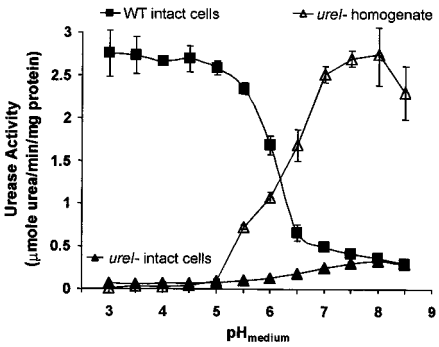

Fig. 2. Comparison of the pH profiles of cytoplasmic urease activity in wild-type (WT) and UreI$^-$ *H. pylori* ATCC 43504 with that of *ureI*$^-$ lysate (n = 3) (*22*). pH_{medium}, pH of the medium. Error bars indicate ± SEM.

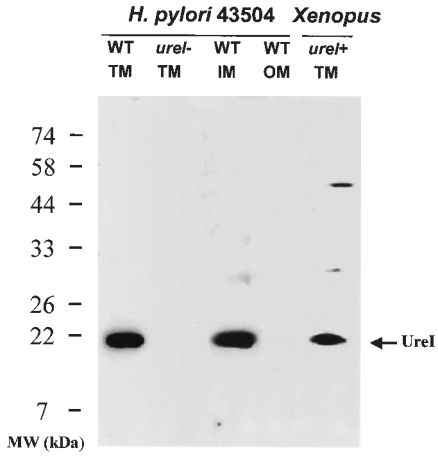

Fig. 3. Western blot analysis showing the absence of UreI in the *ureI* mutants and its presence in the inner but not outer membrane of wild-type *H. pylori*, as well as in oocytes injected with *ureI* cRNA. TM, total membranes; IM, inner membrane; OM, outer membrane (*23*).

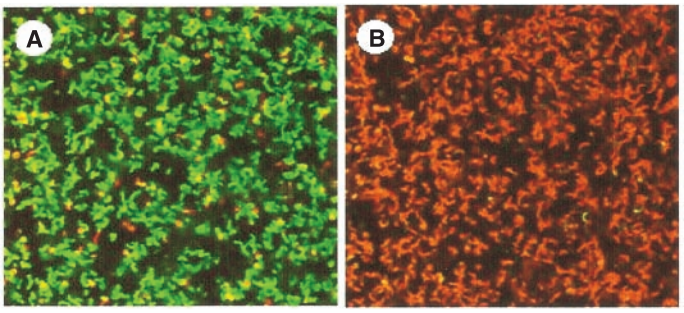

Fig. 1. Confocal fluorescent micrograph of *H. pylori* stained using the Live/Dead method (Molecular Probes, Eugene, Oregon). (**A**) Before $C_{12}E_8$ treatment. Green color is from staining with only SYTO 9, a permeant nucleic acid dye. (**B**) After 0.01% $C_{12}E_8$ treatment. Red stain shows the disrupted membrane that allows entry of propidium iodide.

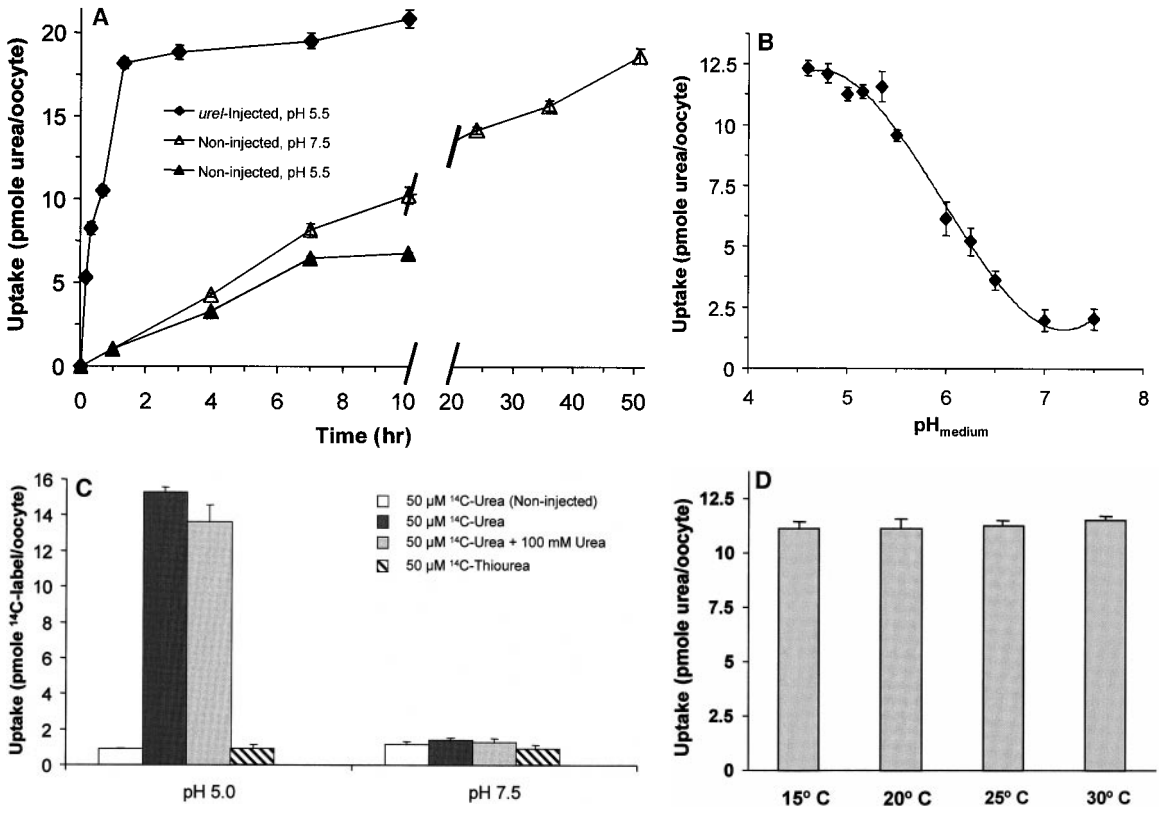

Fig. 4. Uptake experiments in *ureI*-injected and control *Xenopus* oocytes (n = 5 to 7). (**A**) Equilibration of 50 μM ^{14}C-urea at pH 5.5 and 7.5. (**B**) Uptake of 50 μM ^{14}C-urea in *ureI*-injected oocytes as a function of pH of the medium. (**C**) Uptake of 50 μM ^{14}C-urea or uptake of 50 μM ^{14}C-thiourea at pH 5.0 and 7.5 in the presence of excess unlabeled urea. (**D**) Effect of temperature on uptake of ^{14}C-urea at pH 5.0 between 15° and 30°C (*24*). Error bars indicate ± SEM.

follow orientation of translation products in canine microsomal membranes (*19*). A signal anchor sequence translocates the COOH-terminus into the microsomal lumen (analogous to the bacterial periplasm), and a subsequent stop transfer sequence returns the COOH-terminus to the cytoplasmic side. Alternating signal anchor and stop transfer sequences defined (Fig. 5A) the topography of UreI.

The lack of a cytoplasmic retention signal in front of the first hydrophobic sequence, H1, and the presence of two positively charged amino acids in front of H2 imply a periplasmic location of the NH_2-terminus. The COOH-terminus of the UreN1 construct, encoding Met^1–Lys^{23}, was glycosylated, signifying COOH-terminal "out" orientation (Fig. 5A). However, UreN1b, Met^1–Lys^{27}, had a COOH-terminal "in" orientation because of the additional positive charge (*20*). The product of UreN2, Met^1–Thr^{56}, showed strong glycosylation indicating that H1 and H2 are a membrane-inserted pair with the NH_2- and COOH-termini oriented "out." The translation product of UreN3, Met^1–Arg^{102}, lost the glycosylation of UreN2. H3 acts as a stop transfer sequence, yielding a COOH-terminus "in" orientation. The glycosylation of the product of UreN4 translation, Met^1–Leu^{128}, showed that H4 acted as a signal anchor, directing the COOH-terminus "out". The translation product of UreN5, Met^1–Lys^{162}, showed no glycosylation, with H5 acting as a stop transfer sequence, whereas the product of UreN6, Met^1–Val^{195}, was glycosylated. H6 therefore behaved as a signal anchor. Hence, the inner membrane protein, UreI, has six transmembrane–inserted segments, with both NH_2- and COOH-termini located in the periplasm (Fig. 5B).

The apparent pK of UreI activation implies protonation of one or more periplasmic histidines for activation of urea transport. Histidine 123, located at the boundary of H4, was mutated to arginine or glycine (*21*). Expression of UreI was unaffected by this mutation, but acid activation of urea uptake disappeared (1.83 ± 0.20 pmol per oocyte at pH 5.0 versus 1.75 ± 0.34 pmol per oocyte at pH 7.5). The protonated state of this histidine is important for acid activation of transport.

Acid survival of prokaryotes depends on the maintenance of suitable levels of cytoplasmic and periplasmic pH to maintain their PMF. *Helicobacter pylori* survives between pH 4.0 and 8.5 in the absence of urea and grows between pH 6.0 and 8.0 (*2*). A neutral

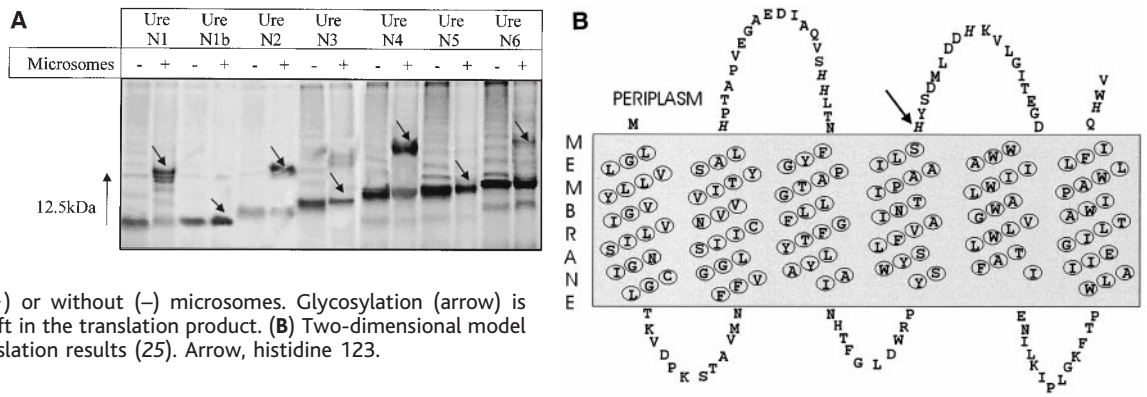

Fig. 5. Topography of UreI. (**A**) SDS–polyacrylamide gel electrophoresis analysis of products resulting from in vitro transcription/translation of successive UreIN-ter/H$^+$,K$^+$-ATPase β-subunit fusion constructs containing one to six of the hydrophobic sequences of UreI, with (+) or without (–) microsomes. Glycosylation (arrow) is detected by a 12.5-kD shift in the translation product. (**B**) Two-dimensional model of UreI from in vitro translation results (*25*). Arrow, histidine 123.

pH–optimum urease must be shielded from gastric acidity and prevented from being active at neutral pH to avoid lethal alkalinization (*5*). Urea transport via UreI allows the internal urease of *H. pylori* to generate ammonia in an acid environment, buffering the periplasm. This allows the organism to survive and grow in the stomach in the presence of usual gastric urea concentrations. The absence of transport by UreI at neutral pH prevents high urease activity in the absence of gastric acidity, as occurs during digestion. The combination of a high level of a neutral pH–optimum urease and an acid-regulated urea channel explains why *H. pylori* is unique in its ability to inhabit the human stomach. Effective inhibition of UreI would provide a means of eradicating the organism in the normal, acid-secreting stomach.

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22. *H. pylori* were grown overnight from glycerol stock on blood agar plates (Baxter, Irvine, CA). *Helicobacter pylori* were disrupted by successive passages through a French pressure cell at 10,000 psi. Urease activity, detected as evolved ^{14}C-CO_2, was measured as previously described (*5*).
23. Antibody generation and affinity purification were carried out by Alpha Diagnostics International (San Antonio, TX). The epitopes UP1 (CEGAEDIAQVSHHLTNFYGPATG) and UP2 (CAILSHYSDMLDDHKVLGITEGD) (*24*) are within the first and second periplasmic loops. Homogenate and membranes were resolved on 10% SDS-tricine gels. Proteins were transferred to either nitrocellulose (Bio-Rad, Hercules, CA) or polyvinylidine difluoride (Millipore, Bedford, MA). After transfer, blots were blocked by incubation in a 5% solution of nonfat dry milk in phosphate-buffered saline–Tween for 1 hour. The membranes were incubated with antibodies to UP2 at a 1:2000 dilution in blocking solution. Binding was detected using a peroxidase-coupled rabbit antibody to immunoglobulin G at 1:20,000 dilution (American Qualex, San Clemente, CA) with ECL or ECL plus (Amersham, Arlington, IL).
24. Reaction buffers contained 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM MES for pH values $\leq$6.0 or 10 mM Hepes for pH values $\geq$6.0. Before each experiment, the oocytes were transferred from Barth's solution to a reaction buffer (pH 7.0) at 21°C. A reaction was started with the transfer of 5 to 7 oocytes to the reaction buffer at 21°C that contained a labeled compound, such as ^{14}C-urea, and was terminated with the transfer of the oocytes to an ice-cold buffer (pH 7.5). Each oocyte was individually dissolved with SDS and mixed with scintillation cocktail for counting of the labeled compounds.
25. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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Transmission of Vesicular Stomatitis Virus from Infected to Noninfected Black Flies Co-Feeding on Nonviremic Deer Mice

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Vesicular stomatitis is an economically important arboviral disease of livestock. Viremia is absent in infected mammalian hosts, and the mechanism by which insects become infected with the causative agents, vesicular stomatitis viruses, remains unknown. Because infected and noninfected insects potentially feed on the same host in nature, infected and noninfected black flies were allowed to feed on the same host. Viremia was not detected in the host after infection by a black fly bite, but because noninfected black flies acquired the virus while co-feeding on the same host with infected black flies, it is concluded that a viremic host is not necessary for an insect to be infected with the virus. Thus co-feeding is a mechanism of infection for an insect-transmitted virus.

Vesicular stomatitis is an arthropod-borne viral disease that primarily affects cattle, swine, and horses; it causes vesicular lesions on the mouth, coronary bands, and teats. Many species of wildlife and humans are also at risk. The causative agents, vesicular stomatitis viruses (VSVs), are a group of antigenically related but distinct viruses of the genus *Vesiculovirus*, family Rhabdoviridae (*1*).

Despite intensive study, aspects of the epizootiology of VSVs, including modes of transmission and endemic maintenance, remain largely unknown and highly controversial. The World Health Organization (WHO) definition of an arbovirus (*2*) implies that only vertebrate species that develop detectable viremia after infection are significant in the epidemiology of these viruses and stipulates that vector infection