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25. Abbreviations for the amino acid residues are A, Ala; D, Asp; E, Glu; F, Phe; G, Gly, H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.
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27. We thank P. Kenny and C. Costello for mass spectroscopic analysis and K. Nakanishi, Y. Lin, and Y. Horikawa for helpful discussions.

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Novel Enzymic Hydrolytic Dehalogenation of a Chlorinated Aromatic

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Microbial enzyme systems may be used in the biodegradation of persistent environmental pollutants. The three polypeptide components of one such system, the 4-chlorobenzoate dehalogenase system, have been isolated, and the chemical steps of the 4-hydroxybenzoate–forming reaction that they catalyze have been identified. The genes contained within a 4.5-kilobase *Pseudomonas* sp. strain CBS3 chromosomal DNA fragment that encode dehalogenase activity were selectively expressed in transformed *Escherichia coli*. Oligonucleotide sequencing revealed a stretch of homology between the 57-kilodalton (kD) polypeptide and several magnesium adenosine triphosphate (MgATP)–cleaving enzymes that allowed MgATP and coenzyme A (CoA) to be identified as the dehalogenase cosubstrate and cofactor, respectively. The dehalogenase activity arises from two components, a 4-chlorobenzoate:CoA ligase–dehalogenase (an αβ dimer of the 57- and 30-kD polypeptides) and a thioesterase (the 16-kD polypeptide).

CHLOROAROMATICS RANK AMONG the most prevalent and environmentally persistent class of synthetic chemicals. Because standard methodologies have proved ineffective in removing these pollutants from the environment, other strategies are evolving. One strategy involves the isolation of microbes that, through a catabolic process, can transform these compounds to harmless end products. Microbial biodegradation of chloroaromatics (*1*) is, however, complicated in that the chlorine atoms interfere with the enzymatic breakdown of the aromatic ring. Some strains of bacteria have overcome this problem by incorporating a dehalogenation step at the start of the catabolic pathway.

Studies have shown that 4-chlorobenzoate (4-CBA) dehalogenase activity exists in *Pseudomonas* sp. strain CBS3 (*2*), *Nocardia* sp. (*3*), *Alcaligenes* sp. *NTP-1* (*4*), and *Arthrobacter* sp. (*5*). These bacteria were isolated from soil based on their ability to survive on 4-CBA as a sole carbon source. In each of these microbes, 4-CBA is first converted to 4-hydroxybenzoate (4-HBA), which in turn is degraded by the enzymes of the protocatechuate branch of the β-keto-adipate pathway. Substrates labeled with ^{18}O, in conjunction with crude protein powders, were used to demonstrate that in *Pseudomonas* sp. strain CBS3 (*6*) and *Arthrobacter* sp. (*7*) the conversion of 4-CBA to 4-HBA occurs by a hydrolytic process (Eq. 1), as opposed to an oxidative one. This observation suggested to us that the 4-CBA dehalogenase of these strains catalyzes a novel aromatic substitution reaction, one that conceivably might be expanded, through active site modification, to other halogenated aromatic substrates. We report the purification and characterization of an enzyme capable of catalyzing hydrolytic dechlorination of chlorinated aromatics.

Earlier attempts to purify active 4-CBA

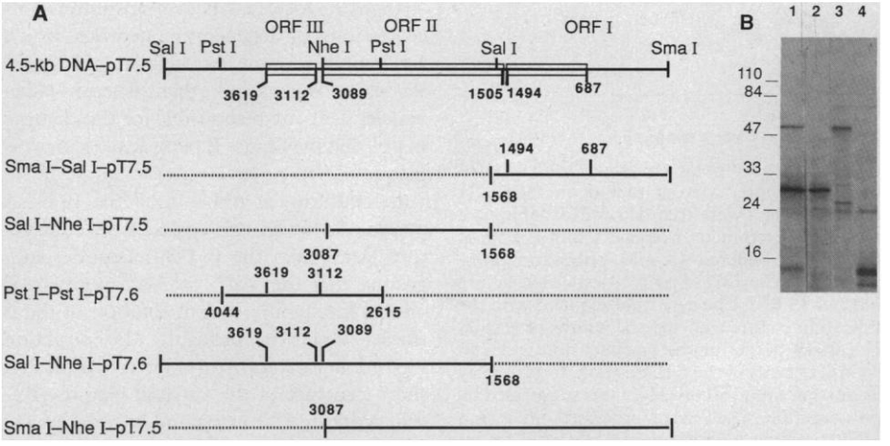

Fig. 1. (**A**) Construction of the pT7.5 clones and pT7.5 and pT7.6 subclones from the 4.5-kb fragment encoding the 4-CBA dehalogenase derived from the chromosomal DNA of *Pseudomonas* sp. strain CBS3. We generated the subclones by, and named them according to, the restriction enzymes indicated. The positions of the open reading frames ORF I, ORF II, and ORF III (identified with oligonucleotide sequencing) that encode the 30-, 57-, and 16-kD polypeptides are shown. (**B**) The autoradiogram of a 12% SDS-PAGE chromatogram of soluble cellular proteins produced by the *E. coli* k38 treated with [^{35}S]Met. Lanes are as follows: pGP1-2 and the 4.5-kb DNA-pT7.5 (lane 1), the Sma I–Sal I–pT7.5 digest (lane 2), the Sal I–Nhe I–pT7.5 digest (lane 3), or the Pst I–Pst I–pT7.6 digest (lane 4). The running positions of commercial stained molecular mass markers are indicated at left in kilodaltons.

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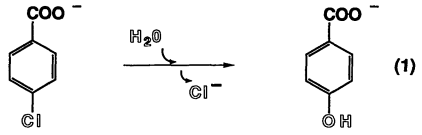


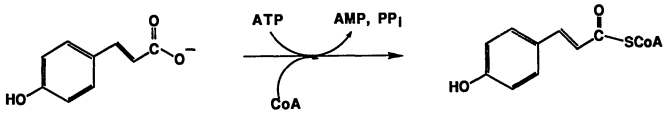
dehalogenase from the *Pseudomonas* sp. strain CBS3 (*8*), *Nocardia* sp. (*3*), *Alcaligenes* sp. *NTP-1* (*4*), and *Arthrobacter* sp. (*5*) had been unsuccessful because of low levels in cell-free extracts of the activity that converts 4-CBA to 4-HBA. We increased this enzymatic activity by enhancing the expression of the dehalogenase genes. Strain CBS3 dehalogenase genes were cloned into *E. coli* with the cosmid vector pPSA843 (*9*). Transfer of the hybrid cosmid carrying a 9.8-kb DNA fragment from strain CBS3 to the 4-CBA dehalogenase–negative strain *Pseudomonas putida* KT2440 conferred the ability to dehalogenate 4-CBA on this strain and enabled the strain to grow on 4-CBA as its sole source of carbon. However, as with the *Pseudomonas* sp. strain CBS3, the 9.8-kb pPSA843 *P. putida* clone yielded cell-free extracts that did not appear to contain sufficient amounts of the converting activity to allow further fractionation. The low and often transient dehalogenase activity observed with lysed cells might have been the result of the dilution or degradation of an unidentified cosubstrate or cofactor by the cellular extract. Less desirable was the possibility that the dehalogenase itself is unstable outside of the intact cell. In view of these obstacles to purification of the dehalogenase activity, we set out to identify the polypeptide components of the 4-CBA dehalogenase through oligonucleotide sequencing and selective expression of the dehalogenase genes.

In order to perform the gene sequencing and expression experiments, we trimmed the 9.8-kb DNA fragment (*9*) with the exonuclease Bal 31 and then cut the DNA with Sma I to produce a smaller, 4.5-kb DNA fragment that, when cloned in pMMB22, transferred 4-CBA dehalogenase activity to *E. coli* (*10*). We examined the protein components of the dehalogenase encoded by the 4.5-kb fragment by using the T7 RNA polymerase–dependent in vivo transcrip tion-translation system for the exclusive expression of the cloned genes. Transformants with plasmid pT7.5 containing the 4.5-kb insert (Fig. 1A) were labeled with [^{35}S]Met (*11*), and the translation products were visualized from the autoradiogram taken of a 12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) chromatogram (Fig. 1B). Lane 1 (Fig. 1B) revealed three polypeptides (57, 30, and 16 kD) as possible candidates for the components of the dehalogenase system. The molecular masses of these translation products were checked against the open reading frames (ORFs) identified by the oligonucleotide sequencing (*12–14*) of the 4.5-kb insert. One ORF spans oligonucleotide positions 687 to 1,494 and encodes a 29,847-dalton polypeptide. A second spans positions 1,505 to 3,089 and encodes a 57,156-dalton polypeptide, and a third spans positions 3,112 to 3,619 and encodes a 19,359-dalton polypeptide.

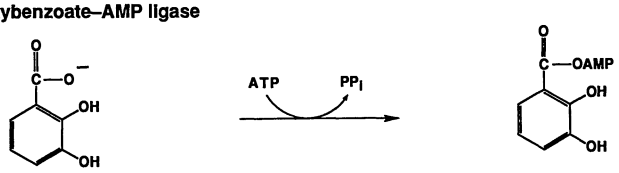
In order to test both the assignment of the ORFs and identify which of the three polypeptides were required for whole-cell dehalogenase activity, we generated fragments of the 4.5-kb oligonucleotide insert that encoded selected ORFs (Fig. 1A) and then separately inserted them into plasmids pT7.5 or pT7.6. The Sma I–Sal I endonuclease fragment, thought to contain ORF I, does in fact encode the 30-kD polypeptide (lane 2, Fig. 1B). Lane 3 (Fig. 1B) shows the polypeptide translation product corresponding to ORF II contained in the Sal I–Nhe I subclone. Shown in lane 4 is the translation product of ORF III contained in the Pst I–Pst I subclone. Hence, the ORFs identified by oligonucleotide sequencing (*12–14*) were confirmed.

The dehalogenase activities of the 4.5-kb DNA–pT7.5 *E. coli* clone and of each of the five *E. coli* pT7.5 subclones represented in Fig. 1A were assayed [reversed-phase high-pressure liquid chromatography (HPLC) separation of 4-CBA and 4-HBA] with whole cells (20 mg of cells per milliliter in 1 mM of 4-CBA and 25 mM tris-HCl, pH 7.5, at 30°C, overnight). Whereas complete conversion of 4-CBA to 4-HBA had taken place in the presence of the cells containing the 4.5-kb DNA–pT7.5 *E. coli* clone, no conversion was observed for the cells con-

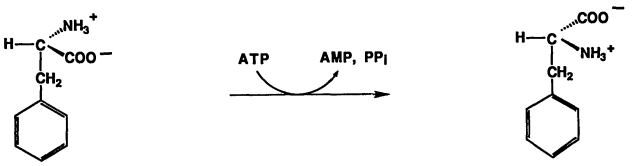
4-Coumarate:CoA ligase



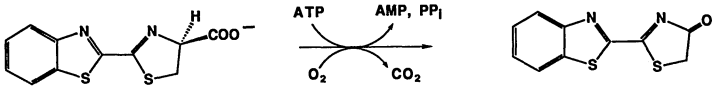
2,3-Dihydroxybenzoate–AMP ligase



Gramicidin S synthetase I and tyrocidine synthetase I



Luciferase



Scheme 1

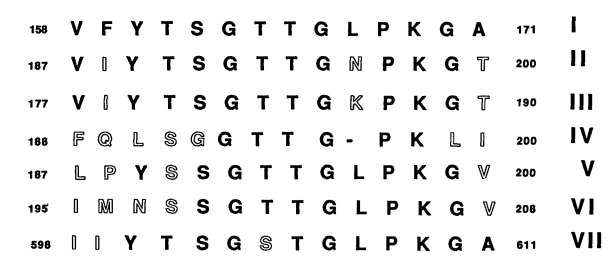
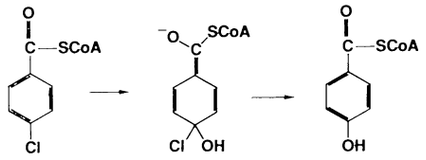


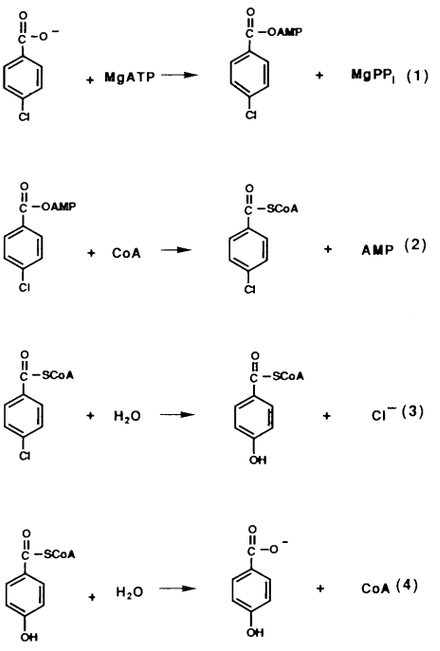
Fig. 2. The conserved stretch of amino acid sequence identified in the 57-kD polypeptide of the 4-CBA dehalogenase (**I**) and the enzymes *Bacillus brevis* gramicidin S synthetase I (**II**) *Bacillus brevis* tyrocidine synthetase I (**III**), *Petroselinum crispum* coumarate CoA:ligase (**IV**), *Photinus pyralis* luciferase (**V**), *E. coli* 2,3-dihydroxybenzoate–AMP ligase (**VI**), and *Vibrio anguillarium* AngR protein (**VII**) (*15–20*). The 57-kD polypeptide sequence was compared with protein sequences contained in the NBRF database (release number 26) with the programs in the EuGene sequence package (*27*). 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.



Scheme 2

taining the subclones. Thus, all three polypeptide components encoded by the 4.5-kb oligonucleotide fragment are required for the 4-CBA to 4-HBA converting activity.

The primary structure of the protein components of the 4-CBA dehalogenase provided the clues for understanding why lysed (as opposed to whole) cells were unable to convert 4-CBA to 4-HBA. We compared the primary structure of the dehalogenase to those of a select group of other proteins. A comparison of the amino acid sequence of the three polypeptides with other protein sequences contained in the National Biomedical Research Foundation (NBRF) database (release number 26) led to the identification of six proteins (*15–20*) that share a common stretch of sequence with the 57-kD polypeptide (Fig. 2). The reactions catalyzed by five of these proteins (gramicidin S synthetase I and tyrocidine synthetase I of *Bacillus brevis*, 2,3-dihydroxybenzoate–adenosine monophosphate (AMP) ligase of *E. coli*, luciferase of *Photinus pyralis*, and 4-coumarate:coenzyme A (CoA) ligase of *Petroselinum crispum*) are shown in Scheme 1 (PPi, inorganic pyrophosphate). The sixth protein, AngR, is thought to be a DNA-binding protein that modulates Fe^{2+}-regulated transcription (*20*) in *Vibrio anguillarum*.



Scheme 3

Each of the enzymes represented in Scheme 1 catalyzes the adenylylation of a carboxylic acid substrate with magnesium adenosine 5′-triphosphate (MgATP) (*21–25*). In the case of the 4-coumarate:CoA ligase reaction, formation of the mixed anhydride precedes acyl transfer to the thiol group of CoA (*21*). The (2,3-dihydroxybenzoyl)adenylate, formed by the 2,3-dihydroxybenzoate–AMP ligase of the *E. coli* enterobactin biosynthetic pathway, ultimately undergoes acyl transfer to the amino group of Ser (*22*). In the reactions catalyzed by gramicidin S synthetase I (*23*), tyrocidine synthetase I (*24*), and (presumably) luciferase (*25*) acyl transfer from the mixed anhydride to a Cys sulfhydryl at the active site occurs in preparation for carbanion formation at the α carbon.

By relating these modes of catalysis to that of the 4-CBA dehalogenase, one can envision a nucleophilic substitution reaction mechanism where delocalization in the negatively charged intermediate, resulting from the attack of water or a hydroxide ion at C-4 of the aromatic ring, is aided by a thioester substituent at C-1 (Scheme 2). The thioester substituent would originate by adenylylation of the carboxyl moiety of 4-CBA, followed by displacement of the AMP with a thiol group from the active site.

We tested the dehalogenase mechanism shown in Scheme 2 by assaying dehalogenase activity in cell-free preparations using ATP (5 mM), Mg^{2+} (5 mM), and 4-CBA (1 mM) in the assay mixture. In this case, we observed stable dehalogenase activity; 36 mU/mg of protein was measured at pH 7.5 and 30°C for fractionated soluble cell protein (a 0 to 80% ammonium sulfate protein fraction of the 4.5-kb DNA pMMB22 *E. coli* clone cellular extract). We then attempted to purify the dehalogenase by column chromatography and failed to observe dehalogenase activity (as assayed by reversed-phase HPLC separation of 4-CBA and 4-HBA) in individual and pooled column fractions, so we then searched for another missing component of the dehalogenase reaction. In view of the possibility that a thiol cofactor, rather than an enzyme active-site Cys, might function in thioester formation from the adenylated 4-CBA intermediate, we screened naturally occurring thiols (Cys, glutathione, and CoA) for their ability to support dehalogenase activity in pooled column fractions; CoA was discovered to be the missing cofactor required for dehalogenase activity. The 4-CBA dehalogenase reaction thus was formulated as the sum of the four partial reactions shown in Scheme 3.

The elution profile obtained after DEAE-cellulose column chromatography of the 0 to 55% ammonium sulfate cut of the soluble protein obtained from the *E. coli* clone 4.5-kb DNA pMMB22 is shown in Fig. 3A. The dehalogenase activity separated into two components. The first component was eluted by buffer, whereas the second component eluted after the application of a KCl gradient. Component I was further purified on a Superose 12 (Pharmacia) fast protein liquid chromatography (FPLC) column that was calibrated with protein molecular mass standards. SDS-PAGE analysis of the ~80-kD native enzyme revealed it to be an αβ

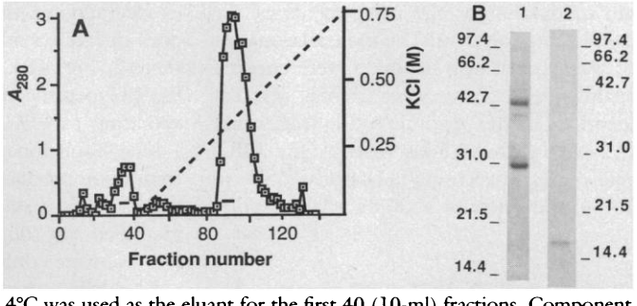


Fig. 3. (**A**) Elution profile of a 0 to 55% ammonium sulfate protein fraction (1 g) taken from *E. coli* cells containing the 4.5-kb DNA pMMB22 plasmid and applied to a 2.5 cm by 50 cm DEAE-cellulose column. An isocratic buffer solution [50 mM Hepes, 5 mM dithiothreitol, and 10 μM $Fe(NH_4)_2(SO)_2$, pH 7.5] at 4°C was used as the eluant for the first 40 (10-ml) fractions. Component I was eluted in fractions 32 to 37. After applying a linear 0 to 0.75 M KCl gradient (800 ml), we eluted the second component in fractions 85 to 89. Protein concentration was measured by the absorbance at 280 nm (A_{280}). Fractions were assayed for the 4-CBA to 4-HBA converting activity in solutions containing 3 mM $MgCl_2$, 3 mM ATP, 0.1 mM CoA, and 25 mM tris-HCl (pH 7.5) at 30°C. We monitored substrate consumption and product formation by injecting aliquots of the reaction onto an analytical C_{18}–reversed-phase HPLC column calibrated with 4-CBA and 4-HBA. Reaction components were eluted with a 50:50:1 methanol:water:acetic acid solution and detected at 254 nm with an ultraviolet detector. We also assayed column fractions for dehalogenase activity by including *p*-hydroxybenzoate hydroxylase (3 units per milliliter) (E.C. number 1.14.13.2), 0.1 mM flavin-adenine dinucleotide, and 0.1 mM dihydronicotinamide adenine dinucleotide phosphate (NADPH) in the assay solution. We monitored the formation of 4-HBA by measuring NADPH consumption at 340 nm with an ultraviolet-visible spectrophotometer. (**B**) A 12% SDS-PAGE chromatogram of pure 4-CBA dehalogenase component I (lane 1) and component II (lane 2) that was stained with Coomassie blue. The running positions of commercial molecular mass markers are indicated in kilodaltons.

dimer of the 57- and 30-kD polypeptides (Fig. 3B). The catalytic properties of purified component I were examined by ^{31}P nuclear magnetic resonance and reversed-phase HPLC. In the presence of Mg^{2+}, CoA, and 4-CBA, component I catalyzes the cleavage of ATP to AMP and PP_i coupled with the formation of the 4-HBA:CoA adduct (the partial reactions 1, 2, and 3 of Scheme 3). Catalysis of ATP cleavage (partial reaction 1, Scheme 3) did not occur in the absence of CoA. Component I, in combination with component II, Mg^{2+}, and CoA, gave complete conversion of 4-CBA and ATP to 4-HBA, AMP, and PP_i.

Component II was purified from the Pst I–Pst I–pT7.6 *E. coli* subclone (Fig. 1A). Fractionation of the 40 to 70% ammonium sulfate protein cut on a DEAE-cellulose column, followed by gel filtration on a calibrated FPLC Superose 12 column, provided pure component II (Fig. 3B). Component II is a ~65-kD protein (α_4 tetramer) that catalyzes the hydrolysis of synthetic 4-HBA:CoA thioester (*26*) to 4-HBA and CoA.

The 4-CBA dehalogenase activity is thus a sum of the activities of a 4-CBA:CoA ligase, a 4-CBA:CoA dehalogenase, and a 4-HBA:CoA thioesterase. We note the role that the dehalogenase sequence data played in the isolation of the active enzyme; even the short motif shown in Fig. 2 allowed us to infer the cosubstrate and cofactor for this reaction. We expect that, as protein sequence databases expand, discoveries of this type could become routine and the characterization of new sequences could be facilitated.

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12. The 4.5-kb insert was sequenced as two separate fragments. A Sal I–Sal I 3.0-kb fragment was cloned into pUC19. Sequencing clones were generated from this plasmid by the method of nested deletions (*13*) (Amersham kit) and analyzed by restriction digest and agarose gel electrophoresis. We performed oligonucleotide sequencing by using the dideoxy chain termination method with the modified form of T7 DNA polymerase (*14*) and [^{35}S]deoxyadenosine triphosphate (dATP) (Sequenase kit of U.S. Biochemical Corp.). Universal and synthetic oligonucleotides were used as primers. The 1.5-kb Sma I–Sal I fragment was cut with restriction endonucleases into four overlapping fragments. The fragments were cloned into M13 mp18 or M13 mp19. We sequenced the oligonucleotides by using the dideoxy chain termination method (Amersham M13 sequencing kit) with [^{35}S]dATP. The nucleotide positions assigned to ORF I (30-kD polypeptide) and ORF II (57-kD polypeptide) have been confirmed by NH_2-terminal peptide sequencing of the purified translation products. The nucleotide positions of ORF III (19-kD polypeptide) await confirmation or adjustment based on the NH_2-terminal sequence of the observed 16-kD translation product.
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Regulatory Role of Parasites: Impact on Host Population Shifts with Resource Availability

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Effects of infections by the ciliate *Lambornella clarki* on larval populations of its mosquito host *Aedes sierrensis* were examined in laboratory and field studies. When host populations developed with sufficient food, mortality from parasites was additive and reduced the number of emerging mosquitoes. For food-limited populations, mortality was compensatory or depensatory; emerging adults were as or more abundant with higher average fitness than those from uninfected control populations. When nutrients were scarce, parasitic infections relaxed larval competition and increased per capita food by reducing host abundance. Food limitation altered larval feeding behavior, reducing horizontal transmission and subsequent mortality from parasitism.

DESPITE THE WIDESPREAD OCCURrence of parasites and the diseases they cause, few quantitative data are available on how these organisms affect host abundance in nature (*1*). Assessing whether parasites regulate host populations is challenging because their impact cannot be inferred from incidence rates alone and because comparative evidence from infected and non-infected populations is extraordinarily difficult to obtain; consequently, models describing the effects of parasites on host population dynamics have relied heavily on laboratory studies (*1*). Theoretically, parasitism can result in a reduction, no change, or even an increase in host abundance; such host mortality effects are termed additive, compensatory, and depensatory, respectively. Determining the frequency and importance of these host population responses in nature and their underlying mechanisms are critical and formidable tasks for both theoretical and applied ecologists (*1*). Moreover, understanding these mortality patterns is crucial for implementing effective biological control strategies.

Despite these difficulties, by creating host and parasite populations in laboratory microcosms and manipulated natural habitats, we have demonstrated that the impact of fatal infections by the parasitic protozoan, *Lambornella clarki* (Ciliophora: Tetrahymenidae), inflicted on populations of its natural mosquito host, *Aedes sierrensis* (Diptera: Culicidae), changes with different resource regimes. Interactions between the effects of food limitation and parasitism as

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