dynamics simulation was reported recently by H. Treutlein *et al.* [in *Photosynthetic Bacterial Reaction Centers: Structure and Dynamics*, J. Breton and A. Vermeglio, Eds. (Plenum, New York, 1988), p. 139]. However, the calculated ΔV did not reflect the relevant electrostatic energy and no rate constant was calculated.

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18. The dispersed polaron model is valid beyond the range of simple harmonic systems. Equation 4 reflects a quasi-harmonic approximation that uses harmonic wave functions as a basis set for anharmonic systems, by adjusting the harmonic frequencies according to a varitaion principle. [For a related treatment, see R. A. Friesner and R. M. Levy, *J. Chem. Phys.* **80**, 4488 (1984).] The present approach ensures that the intersection of V_a and V_b corresponds to the classical anharmonic activation energy, $\Delta g^{\ddagger}$. One could improve this approximation by an explicit consideration of the fact that both $\Delta g^{\ddagger}$ and λ are functions of temperature.
19. The use of ΔG^0 in Eq. 4 (rather than the potential difference between the minima of state a and state b) is particularly important in simulations of reactions with high activation barriers at high temperatures (*12, 13*). In simulations of fast relaxation processes such as the reaction $H^-Q \rightarrow HQ^-$, one really needs the value of $\langle \Delta V_{ba} \rangle_a$. This value requires long dynamics trajectories and a reliable estimate of the gas-phase free energy change (the α^i of Eq. 5). However, ΔG^0 and λ can be used in an approximate way to obtain $\langle \Delta V_{ba} \rangle_a$. In fact, one expects $\langle \Delta V_{ba} \rangle_a$ to change with time in a temperature-dependent manner as the system relaxes after the electron transfer [see (*21*) for related experimental information on the H^-Q system]. Such relaxations could be studied by direct simulations, but these would have to be longer than the one considered here.
20. The potential parameters used in Eq. 4 were the same as those used previously (*4*). We also evaluated the intramolecular gas-phase energy of forming HQ^- from H^-Q at infinite separation ($\alpha^b - \alpha^a$), following the approach described in (*4*). The starting coordinates for the calculations were taken from the x-ray structure (*1*) of *Rps. viridis* and included the atoms of P, H, and Q, the non-heme iron, and all amino acid residues within 18 Å of the electron D or A (either H and Q or Q and P, depending on the reaction). The calculations also allowed water molecules to fill any cavities within this region of the protein (*4*). All potentially ionizable amino acid residues were taken to be in their neutral forms; as argued by A. Warshel and S. Russell [*Q. Rev. Biophys.* **17**, 283 (1984)], the effective dielectric constant for ionized groups in proteins is large. The atomic charges for the electron carriers in their initial and final states were evaluated by the QM Consistent Force Field for π electron systems (QCFF/PI) method with the parameters taken from the following: A. Warshel and A. Lappicirella, *J. Am. Chem. Soc.* **103**, 4464 (1981); A. Warshel and W. W. Parson, *ibid.* **109**, 6143 (1987). We obtained the intramolecular potential surface V^i_{strain} by using a classical force field that approximated the corresponding QCFF/PI surface, representing the change of the potential between different redox states in terms of shifted harmonic potentials. [Intramolecular Franck-Condon factors for these shifted surfaces have been evaluated (*11, 12*).] The non-heme iron atom, which is well solvated by nearby glutamic acid and histidine residues, was assigned an effective charge of +0.2. The region of the protein that was treated in detail was assumed to be surrounded by a continuum with a dielectric constant of 2 to represent the membrane and the rest of the protein.

    The calculations were done by the program ENZYMIX, running a 20-ps trajectory at 300 K on V_a and recording the energy gap $\Delta V_{ba}(t) = V_b(t) - V_a(t)$ for the last 10 ps. (During the first few picoseconds, the molecular structure relaxes very rapidly toward an energy minimum for the state P^+H^-. This initial relaxation probably is not relevant to the $H^-Q \rightarrow HQ^-$ reaction, which occurs on the time scale of 200 ps.) The time-dependent energy gap during the second period (Fig. 1) was used to obtain the frequency-dependent origin shifts, $\Delta(\omega)$, of Fig. 4 by a Fourier transform procedure (*13*).
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Symbiotic Marine Bacteria Chemically Defend Crustacean Embryos from a Pathogenic Fungus

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Embryos of the shrimp *Palaemon macrodactylus* are remarkably resistant to infection by the fungus *Lagenidium callinectes*, a recognized pathogen of many crustaceans. An *Alteromonas* sp. bacterial strain consistently isolated from the surface of the embryos, produces 2,3-indolinedione (isatin), a compound that inhibits the pathogenic fungus. If exposed to the fungus, bacteria-free embryos quickly die, whereas similar embryos reinoculated with the bacteria or treated only with 2,3-indolinedione live well. The commensal *Alteromonas* sp. bacteria protect shrimp embryos from fungal infection by producing and liberating the antifungal metabolite 2,3-indolinedione.

THE PRODUCTION OF SECONDARY metabolites, or allelochemicals (*1*), by microbes is thought to be an important adaptation allowing microbes to affect their hosts, competitors, and predators (*2–4*). However, it is difficult to extrapolate laboratory experiments to the complex interactions of microbes in nature (*5*) and few rigorous tests of these ideas have been conducted with ecologically relevant organisms. Thus, the chemical ecology of microorganisms has remained a largely hypothetical field (*6*), and one in need of new research approaches to confirm the natural functions of microbial metabolites.

Many organisms remain free of pathogenic fungi and bacteria as long as they retain a specific group of microorganisms that constitute normal associated flora (*7–9*) on their surface. In some instances, epibiotic microorganisms produce compounds that inhibit competing microorganisms (*10–12*). It has been suggested that commensal microorganisms may chemically defend their microhabitat, thus providing their host protection from pathogenic microorganisms (*13, 14*). Our research confirms that antimicrobial compounds produced by marine bacteria are important in microbial competition and host viability.

Although juveniles and larvae of the caridean shrimp *Palaemon macrodactylus* are highly vulnerable to infection by the phycomycetous fungus *Lagenidium callinectes* (*15*) [a pathogen of many crustaceans (*16–18*)], externally brooded embryos of are remarkably resistant to the fungus. Fisher (*19*) isolated several bacterial strains from the surface of healthy *P. macrodactylus* embryos and noted that the embryos were quickly colonized by a variety of different microorganisms when their associated bacteria were removed by treatment with penicillin. The fungus *L. callinectes* was one of the most frequent and vigorous pathogens (*20*), and infection always resulted in death. A penicillin-sensitive bacterial strain (culture I-2, an *Alteromonas* sp.) that is consistently isolated from healthy embryos was found to effectively inhibit the growth of the fungus *L. callinectes* in vitro. We found that this strain produces, and releases into the culture medium, relatively large quantities of an antifungal compound (*21*). We have isolated and identified this compound as 2,3-indolinedione (*22*) (also known as isatin), a substance previously known mainly as a synthetic intermediate in the production of indigo dyes, but known to possess significant pharmacological properties (*23*) (Fig. 1).

To confirm that the bacterial isolate protects the embryos from fungi and that protection is mediated by 2,3-indolinedione, we performed the following experiment on four groups of embryos. Clusters of embryos were detached from 20 different *P. macrodactylus* females. Each embryo cluster was divided in four groups of about 30 embryos each (*24*). The epibiotic bacteria were eliminated from three of these groups by treatment with penicillin-G (*25*). One group

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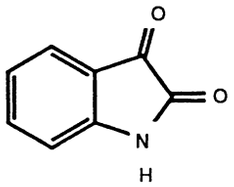

Fig. 1. The molecular structure of 2,3-indolinedione.

received no other treatment after the initial penicillin wash. The second group was reinfected with pure cultures of the associated bacterial strain I-2 (*26*). The third group was dipped periodically in solutions of 2,3-indolinedione (*27*). The three groups treated with penicillin were then infected with thick liquid cultures of the pathogenic fungus *L. callinectes*. Representatives of each embryo group, including those from a fourth group that received no treatment (that is, control), were observed under the microscope daily and two or three of the typical embryos were fixed for standard electron microscopy examination (Fig. 2).

Colonization of the embryonal surface by the fungus *L. callinectes* could be observed after 3 days. Each day thereafter, each isolate of 30 embryos was classified as either alive, some alive, or all dead. Survival of replicate embryo groups (Fig. 2) in the penicillin-only treatment was 5% (*28*); penicillin-treated embryos reinfected with pure bacterial culture I-2 or treated with 2,3-indolinedione had survival rates of approximately 60%. Survival in the control group was 80%. At the end of the 12-day experiment, survivorship of embryos treated with only

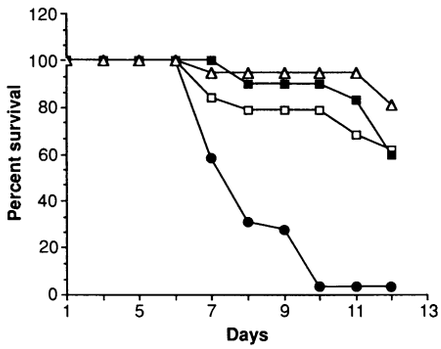

Fig. 3. Percent survival of embryo groups (n = 20 for each treatment). On day 12, survivorship of embryo groups treated only with penicillin (●) differed significantly ($P < 0.001$) from all other treatments; there were no significant differences among the other treatments ($P > 0.1$, 2 × 4 contingency table analysis using a G test). ●, Bacterium removed, no further treatment; □, bacterium removed, then treated with 2,3-indolinedione; ■, bacterium removed, then reintroduced; △, control.

penicillin was significantly ($P < 0.001$, contingency table analysis by the simultaneous test procedure) lower than for all other treatments (Fig. 3); survivorship of penicillin-treated embryos reinfected with bacterial culture I-2 or treated with 2,3-indolinedione did not differ from each other or from the control embryos ($P > 0.10$).

The results of this experiment show that the epibiotic bacterium *Alteromonas* sp. strain I-2, commonly associated with embryos of *P. macrodactylus*, is able to deter infection by the pathogenic fungus *L. callinectes*. The mechanism by which this protection is achieved is the production and release of the antifungal compound 2,3-indolinedione, demonstrating the natural function of a microbial allelochemical. Glover *et al.* (*29*) have isolated 2,3-indolinedione from both human and rat tissues and established that it is the endogenous monoamine oxidase inhibitor known previously as tribulin. The relation of their observations to our recorded antifungal properties is unclear at this point, but each observation points to the previously unrecognized importance of isatin in regulating natural phenomena. These results also have important ecological and evolutionary implications (*14*), the significance of which will become clearer as similar microbial associations are identified and experimentally investigated. These observations suggest that aquatic plants and animals are particularly vulnerable to pathogenic microorganisms because of their constant intimate contact. The adaptation of surface bacterial symbiosis may then represent an essential adaptation in freshwater and marine environments.

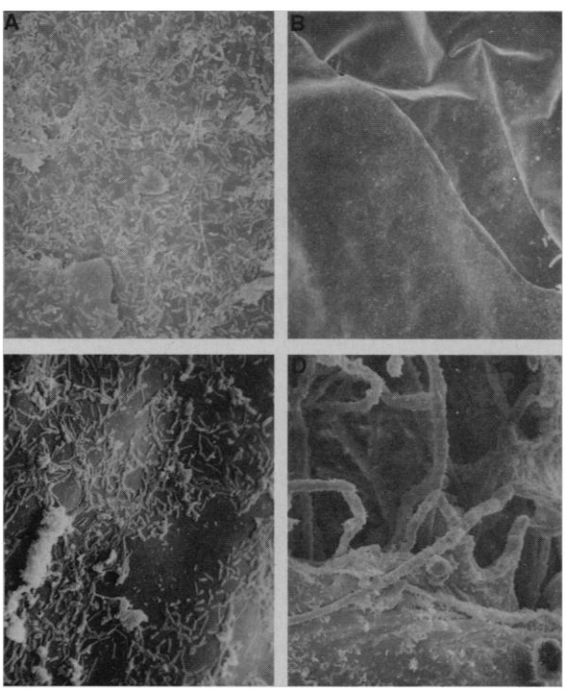

Fig. 2. Scanning electron micrographs of representative embryos during the experiment. (**A**) Bacteria on surface of healthy embryo (×2000). (**B**) Surface of embryo after treatment with penicillin-G (×1000). Although in some instances a few large bacteria could be seen on the embryos, most of the bacterial population was eliminated. (**C**) Embryos after recolonization with pure cultures of the protective bacterium (×2000). (**D**) Hyphae of the fungus *L. callinectes* penetrating the embryonal coat (×1000).

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21. *Alteromonas* sp. (strain I-2) was cultured in 16-liter carboys with aeration for 2 weeks (23°C). The culture medium consisted of 5 g of bactopeptone, 3 g of yeast extract, and 10 g of starch per liter of seawater. The antifungal metabolite was isolated (15 mg/liter) by extraction of the medium with ethyl acetate after 2 weeks.
22. The active compound 2,3-indolinedione was purified by silica flash chromatography (with 70% ethyl acetate–isooctane) of the crude extract, followed by high-performance liquid chromotography (60% ethyl acetate–isooctane on a silica gel (μ-Porasil column). The active metabolite was characterized as 2,3-indolinedione on the basis of its physical and spectral properties: red-orange prisms, soluble in

methyl alcohol and dimethyl sulfoxide; melting point, 201° to 203°C; infrared: 3200, 3000, 1720 to 1680 band range (br), 1620 to 1550 br cm^{-1}; high-resolution mass spectrometry: requires 147.010 for $C_8H_5NO_2$, found 147.030; ^1H nuclear magnetic resonance, 360 MHz ($CDCl_3$): δ 8.2 (s, 1 H), 7.6 (dd, 1 H, J = 7.7 Hz), 7.5 (d, 1 H, J = 7.8 Hz), 7.1 (dd, 1 H, J = 7.7 Hz), 7.0 (d, 1 H, J = 7.8 Hz). The metabolite isolated was identical in all regards to a sample of 2,3-indolinedione (isatin) obtained from commercial sources.

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24. Each group of embryos was suspended by a thread in a Nalgene centrifuge tube containing 50 ml of autoclaved 50% seawater/pondwater with individual aeration through a Pasteur pipette.
25. Embryos were dipped in a solution (100 mg/liter) of penicillin-G (1600 units per milligram) for 6 hours on the first and second days of the experiment.
26. On day 3 of the experiment embryos were dipped for 2 hours in a liquid culture (medium used was 2216 marine broth) of the bacterium strain I-2.
27. On days 3, 5, and 7 of the experiment embryos were dipped in a solution of 200 μg of 2,3-indolinedione per milliliter for 1 hour.
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30. We appreciate the critical reading and constructive comments provided by N. D. Holland. We are indebted to W. S. Fisher for introducing us to this problem and to W. Clark for providing laboratory space, constructive criticism, and considerable assistance in carrying out this work. This research is a result of financial support provided by the NSF, Chemistry and Oceanography Divisions, under grant CHE86-20217, and in part, from the NIH, National Cancer Institute under grant CA44848.

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A Single Amino Acid Interchange Yields Reciprocal CTL Specificities for HIV-1 gp160

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For the IIIB isolate of human immunodeficiency virus type–1 (HIV-1), the immunodominant determinant of the envelope protein gp160 for cytotoxic T lymphocytes (CTLs) of H-2^d mice is in a region of high sequence variability among HIV-1 isolates. The general requirements for CTL recognition of peptide antigens and the relation of recognition requirements to the natural variation in sequence of the HIV were investigated. For this purpose, a CTL line specific for the homologous segment of the envelope from the MN isolate of HIV-1 and restricted by the same class I major histocompatibility (MHC) molecule (D^d) as the IIIB-specific CTLs was raised from mice immunized with MN-*env*–recombinant vaccinia virus. The IIIB-specific and MN-specific CTLs were completely non–cross-reactive. Reciprocal exchange of a single amino acid between the two peptide sequences, which differed in 6 of 15 residues, led to a complete reversal of the specificity of the peptides in sensitizing targets, such that the IIIB-specific CTLs lysed targets exposed to the singly substituted MN peptide and vice versa. These data indicate the importance of single residues in defining peptide epitopic specificity and have implications for both the effect of immune pressure on selection of viral mutants and the design of effective vaccines.

CYTOTOXIC T LYMPHOCYTES (CD8$^+$) recognize a complex of peptide and class I MHC molecules on the surface of target cells (*1*). With the availability of the crystal structure of a human class I (HLA) molecule (*2*) and the results of structure-function studies of mutant class I molecules and variant peptide antigens (*3, 4*), the structural basis of CTL recognition of these surface molecules is being elucidated. However, the general requirements for an effective peptide antigen and the relation between peptide sequence, MHC binding capacity, and the specificity of recognition by the T cell receptor are still poorly understood. Although the minimal length of antigenic peptides has been well characterized in several studies, the role of individual residues in controlling class I MHC binding and dictating T cell specificity has been carefully examined in only a few model systems (*4*), with variable results in terms of the number of residues critical for each function. Our definition of a minimal peptide determinant of 15 amino acids (RIQRGPGRAFVTIGK, residues 315 to 329) in the HIV-1 gp160 molecule, recognized in the context of H-2D^d (*5*), allowed us to study both the general requirements for peptide antigenicity and the relation between viral sequence variation and immunogenicity of this defined determinant. This was possible because the sequence lies in a segment of the gp160 molecule that shows a high degree of variability among HIV-1 isolates.

We found that we could induce CTLs specific for the immunodominant region when BALB/c mice were immunized with recombinant vaccinia virus carrying the IIIB isolate of human immunodeficiency virus type 1 (HIV-IIIB) gp160 envelope gene and their spleen cells were restimulated with the target peptide (*6*). Therefore, we immunized mice of several haplotypes, such as BALB/c (H-2^d), C3H (H-2^k), and C57BL/6 (H-2^b), with recombinant vaccinia virus expressing gp160 of the MN isolate of HIV-1 (HIV-MN) and restimulated them with interleukin-2 (IL-2) plus a peptide corresponding to the sequence in the HIV-MN gp160 envelope protein homologous to the previously identified CTL epitope of HIV-IIIB (*5*). We found that we could elicit CTLs specific for the homologous region of HIV-MN gp160 (RIHIGPGRAFYTTKN) (18MN) (Table 1) that can also kill in a highly specific manner targets infected with recombinant vaccinia virus expressing whole gp160 MN (Table 2). CTLs were not induced by stimulation in vitro of cells from unimmunized mice with peptide and IL-2, indicating that in vivo immunization was necessary. These MN-specific CTLs are conventional CD4$^-$CD8$^+$ CTLs, because they are killed by treatment with monoclonal antibodies to CD8 plus complement but not by antibody to CD4 plus complement (*6*). The CTLs directed against MN do not kill IIIB or RF recombinant vaccinia virus–infected targets, in agreement with our previous results that a CD4$^-$CD8$^+$ CTL line specific for gp160 IIIB did not cross-reactively kill targets infected with recombinant vaccinia viruses expressing the envelope gene from the RF and MN isolates (*7*). We could induce such variant MN-specific CTLs in BALB/c mice, but could not detect any specific CTLs from C3H and C57BL/6 mice, consistent with our findings using the HIV-IIIB envelope (*5*). Therefore, it was of interest to determine the class I MHC restriction of the newly identified CTL determinant in the MN variant. By using a panel of concanavalin A blast targets, we found that these MN-

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