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NOTES

Eukaryotic Interference with Homoserine Lactone-Mediated Prokaryotic Signalling

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Acylated homoserine lactones (AHLs) play a widespread role in intercellular communication among bacteria. The Australian macroalga *Delisea pulchra* produces secondary metabolites which have structural similarities to AHL molecules. We report here that these metabolites inhibited AHL-controlled processes in prokaryotes. Our results suggest that the interaction between higher organisms and their surface-associated bacteria may be mediated by interference with bacterial regulatory systems.

Acylated homoserine lactones (AHLs) serve as signals in bacterial communication. AHLs and their derivatives regulate bioluminescence, Ti plasmid transfer, production of virulence factors and antibiotics (for reviews, see references 15 and 24), and swarming motility (14). These bacterial processes are fundamental to the interaction of bacteria with each other, their environment, and, particularly, higher organisms. It might therefore be expected that plants or animals would have evolved strategies to interfere with bacterial AHL-mediated processes. The seaweed *Delisea pulchra* (Rhodophyta) produces a number of halogenated furanones (9), which are structurally similar to the bacterial AHLs (Fig. 1) and have strong biological activity (7), including antifouling and antimicrobial properties (8, 23). We hypothesized that these metabolites could interfere with bacterial processes which involve AHL-driven quorum-sensing systems. This hypothesis was tested in terms of responses known to be regulated by AHLs; swarming motility in *Serratia liquefaciens* (14) and bioluminescence produced by the marine bacterial strains *Vibrio fischeri* and *Vibrio harveyi* (15).

Bacterial swarming is a multicellular, density-dependent behavior that is induced in response to recognition of surfaces with a certain viscosity. Cells differentiate into a multinucleated, elongated, and hyperflagellated form, orient themselves lengthwise in close contact with each other, and then move rapidly in a coordinated fashion over the surface of the growth substratum. Swarming has been described for a variety of bacteria, including members of the genera *Serratia*, *Proteus*, *Vibrio*, *Bacillus*, *Escherichia*, and *Salmonella* (1, 2, 17). For *Proteus mirabilis* and *Vibrio parahaemolyticus*, the ability to differentiate into the swarmer cell state plays an important role in bacterial virulence, surface adsorption, and colonization (3–5).

S. liquefaciens is a suitable model organism, because members of the genus can colonize a wide variety of surfaces in water, soil, plants, insects, fish, and humans (16). The formation of a swarming colony of *S. liquefaciens* was recently shown to involve two genetic switches, the first of which encodes a quorum-sensing control mechanism employing at least two extracellular signal molecules, *N*-butanoyl-L-homoserine lactone (BHL) and *N*-hexanoyl-L-homoserine lactone (Fig. 1 and reference 14). The second involves the *flhDC* master operon, which regulates expression of the flagellar regulon and governs control over swim and swarm cell differentiation (13).

Development of a swarming colony can be roughly divided into three stages. In stage 1, the cells grow and establish a normal-sized colony. In stage 2, aligned bundles of elongated, hyperflagellated, highly motile swarm cells emerge at the edge of the dense colony. Following this, in stage 3, the colony spreads rapidly over the surface by means of swarming motility. A swarming colony displays multicellular behavior. The front consists of swarming cells, followed by a thick layer of vegetative cells engaged in growth and cell division. The two layers of cells never separate. Therefore, the velocity at which the colony expands is dependent on the bacterial growth rate as well as the activity of the swarm cells.

Agar plates for induction of swarming motility in *S. liquefaciens* contained minimal AB medium (6) supplemented with 0.4% glucose, 0.5% Casamino Acids, and 0.6% Bacto agar. On this medium, the radius of the swarming colony expands at a rate of approximately 6 mm/h at 30°C. If the medium of an agar plate was supplemented with either of the two *D. pulchra* furanones shown in Fig. 1, the velocity by which the swarming colony expanded was reduced in a concentration-dependent way (Fig. 2 and 3A). The compounds did not affect the formation of a dense colony at the point of inoculation. Once swarming cells had been formed, the *Delisea* compounds caused a reduction in the velocity at which the colony spreads over the surface (Fig. 3A).

A number of processes other than swarming motility per se could be influenced by these compounds. A reduction in bacterial growth rate or general inhibition of motility could account for the observed effect. We therefore monitored growth, from early log phase to stasis, in the presence of the highest

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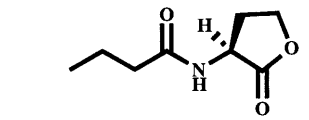
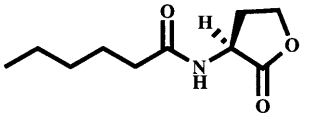
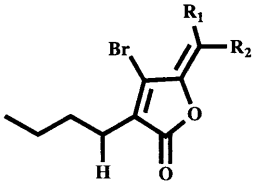


N-butanoyl-L-homoserine lactone (BHL)



N-hexanoyl-L-homoserine lactone (HHL)



	R1	R2
1	Br	Br
2	H	Br

FIG. 1. Structure of AHLs produced by *S. liquefaciens* and two furanones produced by *D. pulchra*.

concentration of both furanone compounds (100 μg/ml). Neither of the compounds had any growth inhibitory effect (see Fig. 6A). Interference with general requirements for motility was investigated by classical swimming of *S. liquefaciens* in soft medium (0.3% Bacto agar) where cells swim through the water-filled channels (Fig. 3B). Bacterial growth and motility lead to the formation of concentric circles of high bacterial density, which move rapidly outward from the point of inoculation. The presence of 100 μg of either furanone per ml in the soft agar did not reduce the outward velocity of the circles, demonstrating that swimming was unaffected by the presence of *D. pulchra* compounds 1 and 2 (Fig. 3B). Swimming motility is, in contrast to swarming motility, an AHL-independent process (14). Our result indicates that the necessary development of flagellation by the swimming cells is unaffected by the two furanones. Furthermore, the fact that the velocity at which the expanding chemotactic rings developed in the 0.3% swim agar is dependent on the growth rate as well as on swimming motility further supports our contention that growth is unaffected by furanones.

The presence of furanone compounds could abolish the FlhDC-controlled developmental process leading to hyperflagellated swarm cells (13). Fewer flagella per cell would then in turn account for the reduced surface motility observed in the presence of the compounds. The flagellar content per cell mass in relation to surface motility was assessed by analyzing cells from the edge of fast-swarming colonies as well as from slowly swarming colonies which were obtained from expanding colonies cultured in the presence of 50 μg of either furanone per ml. Cells from the edge of the colonies were washed off and normalized to the same optical density (OD at 450 nm [OD_{450}] = 1). The amount of flagellar protein in each sample was assessed by a Western blotting (immunoblotting) analysis

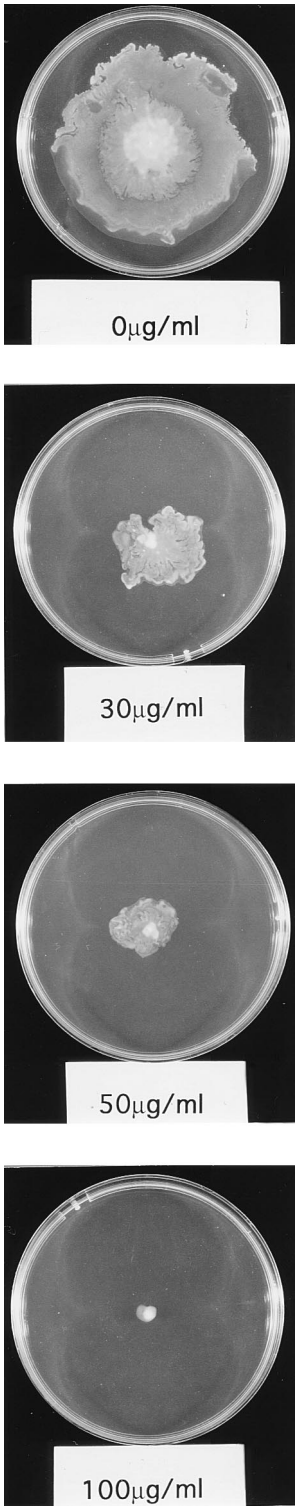


FIG. 2. Effect of increasing concentrations (0, 30, 50, and 100 μg/ml) of *D. pulchra* furanone 2 on *S. liquefaciens* swarming motility. Agar plates were stab inoculated at the center from an exponentially growing culture (OD_{450} of approximately 0.5) and incubated at 30°C. Swarming colonies were photographed 20 h after inoculation.

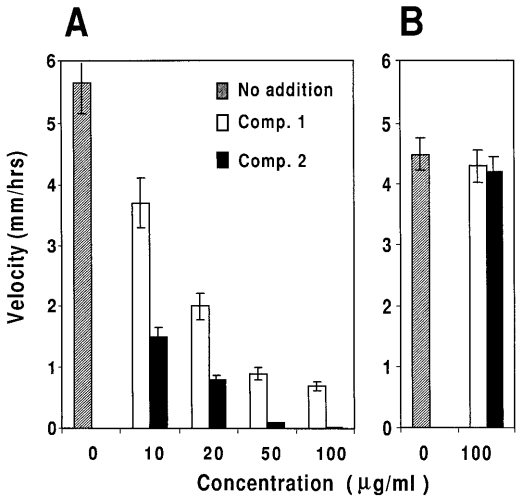


FIG. 3. (A) Velocities at which the front of the swarming *S. liquefaciens* colonies spread over the 0.6% agar surface in the presence or absence of each of the two *D. pulchra* furanone compounds calculated over a 5-h period, beginning 20 h after inoculation. (B) Velocities at which the front of the swimming colonies spread through the 0.3% agar in the presence or absence of the two *D. pulchra* compounds. This was calculated over a 5-h period beginning 20 h after inoculation. Plates were stab inoculated from an exponentially growing culture (OD_{450} of approximately 0.5) at the center of the agar plates and incubated at 30°C. In all cases, the AB medium was supplemented with 0.4% glucose, 0.5% Casamino Acids, and *D. pulchra* compounds (Comp. 1 and 2) as indicated.

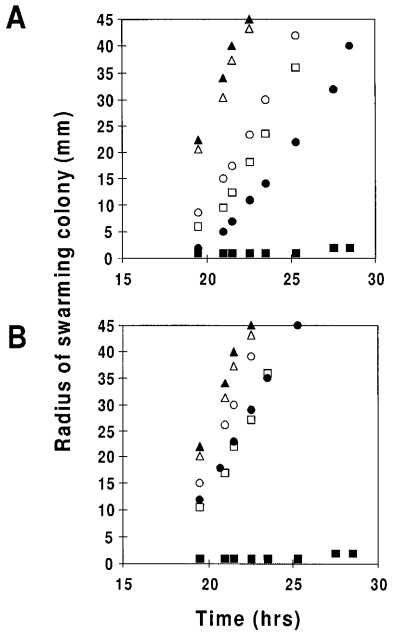


FIG. 5. Competition experiment showing the effect of various concentrations of *D. pulchra* compounds 1 and 2 and BHL on *S. liquefaciens swrI* swarming motility. In panel A, furanone compound 1 was used as an inhibitor, and in panel B, furanone compound 2 was used as an inhibitor. The concentrations used are indicated below. Media, inoculation, and incubation are as described in the legend to Fig. 3. The radii of the swarming *swrI* colonies were measured from 18 to 30 h after inoculation. △, 2 μM BHL; ▲, 20 μM BHL; □, 2 μM BHL + 2 μM compound 1 or 2; ○, 20 μM BHL + 2 μM compound 1 or 2; ■, 2 μM BHL + 20 μM compound 1 or 2; ●, 20 μM BHL + 20 μM compound 1 or 2.

employing antibodies directed against *S. liquefaciens* flagellar protein. The flagellar protein content per cell mass did not differ significantly between cells from fast- and slowly swarming colonies (media supplemented with 50 μg of furanones per ml) or cells from a nonswarming *swrI* colony (Fig. 4). The latter strain is unable to synthesize AHL molecules and therefore is unable to form a swarming colony (14). Microscopic inspections revealed that the cells present in the swarm of the slowly and fast-swarming colonies were equally elongated (not shown). The samples included in lanes f and g were obtained from growing liquid cultures of wild-type (*wt*) and *swrI* strains (OD_{450} = 1) demonstrating the smaller amount of flagellar protein present in cells expressing the swim cell morphology. The *D. pulchra* compounds therefore reduce the motility of the swarm cells by means other than influencing flagellar synthesis, cell elongation, or growth rate.

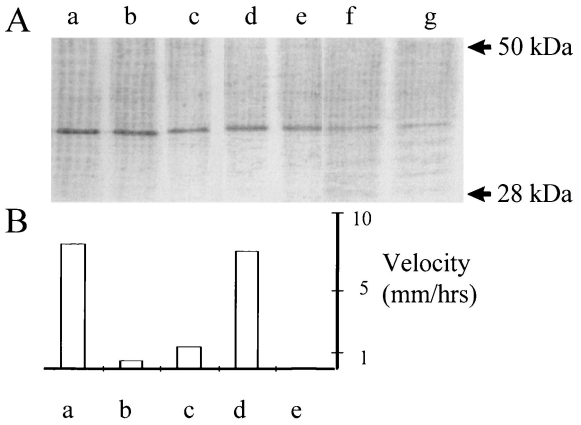


FIG. 4. Content of *S. liquefaciens* flagellar protein. (A) Western blot. Lanes: a and d, fast-swarming *wt* cells; b, slowly swarming *wt* cells in the presence of 50 μg of *D. pulchra* compound 1 per ml; c, slowly swarming *wt* cells in the presence of 50 μg of *D. pulchra* compound 2 per ml; e, nonswarming *swrI* cells; f and g, *wt* and *swrI* cells, respectively, obtained from liquid cultures. (B) Velocities at which the front of the corresponding colonies spread over the surface. Cells from the edge of a colony were washed off in 0.9% NaCl, harvested by centrifugation, and, upon resuspension, normalized to an OD_{450} of 1. Aliquots (50 μl each) were heat denatured in sodium dodecyl sulfate (SDS)-containing sample buffer, and the proteins were then separated by means of a standard SDS-polyacrylamide gel electrophoresis procedure, transferred to an Immobilon-P membrane (Millipore), and subjected to Western blotting analysis involving rabbit antibodies directed against *S. liquefaciens* flagellar protein. After binding of secondary alkaline phosphatase-labelled anti-rabbit immunoglobulin G, detection was performed with *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

Cell-to-cell communication by means of AHL signal molecules plays a crucial role in swarming motility (14). On the basis of a DNA sequence analysis (25), *S. liquefaciens* is expected to encode an AHL receptor protein (a LuxR analog [15]) which we hypothesize functions as a regulator of gene expression. After a threshold value of the AHL-receptor complex has been reached, the autoinduction circuit would be triggered, which, in combination with a suitable surface, leads to formation of motile swarmer cells. Because of the structural similarities between the AHL molecules and the *D. pulchra* furanones, we hypothesized that these compounds affected the interaction between the AHL molecules and the putative regulatory protein by competitively binding to the receptor site. AHL molecules possessing substitutions in the homoserine lactone ring have previously been shown to inhibit bioluminescence in *V. fischeri* (11). Increasing the concentration of BHL in the swarm agar would then be expected to overcome the inhibition. In order to perform such an analysis, we employed the *swrI* mutant of *S. liquefaciens* in which swarming motility is inducible by the addition of BHL to the medium of the 0.6% agar plates. A concentration of 0.2 μM BHL was found to fully

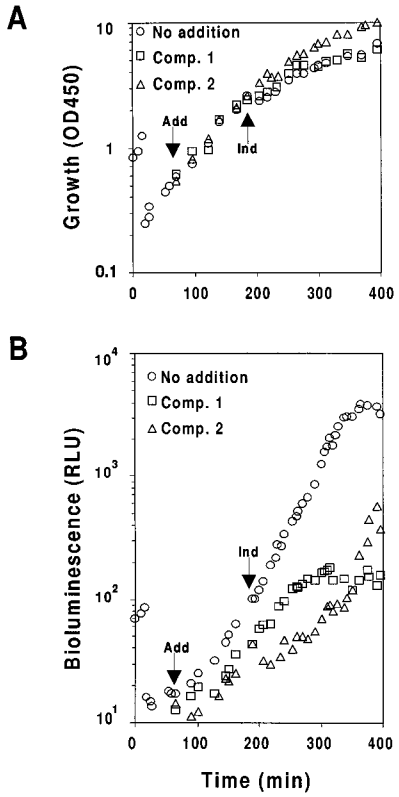


FIG. 6. *S. liquefaciens* cells harboring the bioluminescent AHL monitor plasmid pSB403 were grown at 30°C in AB medium with 0.4% glucose and 0.5% Casamino Acids for approximately 10 generations prior to splitting into three subcultures, two of which were supplemented with either *D. pulchra* compound 1 or 2 (Comp. 1 or 2) at 100 μg/ml. (A) Bacterial growth was measured as OD_{450}. The arrow labelled "Add" indicates the point at which the *D. pulchra* compounds were added. (B) Expression of bioluminescence in the absence or presence of *D. pulchra* compounds 1 and 2. Emission of bioluminescence was measured in a Turner TD-20e luminometer (Turner Designs, Sunnyvale, Calif.). The arrow labelled "Ind" indicates the point at which the specific activity of bioluminescence (relative light units [RLU]/OD_{450}) starts to increase in the unsupplemented culture.

restore swarming to the level of the *wt* strain (14). In the experiments presented in Fig. 5, the concentrations of both BHL and the *D. pulchra* furanone compounds were varied. As judged from the velocity of the expanding colonies, addition of either furanone 1 or 2 at 0.2 μM had no effect in the presence of 0.2 μM BHL (not shown). However, the inhibitory effect of addition of furanones to 2 and 20 μM is reversible by the addition of increasing concentrations of exogenous BHL. This strongly suggests that BHL and the two furanone compounds compete for the receptor site of the putative regulatory protein.

The observed competitive inhibition of bacterial swarming motility suggested that the *D. pulchra* compounds would also inhibit other AHL-regulated phenotypes. We tested this by using the bioluminescent monitor plasmid pSB403, which has proven useful for identifying AHL-producing organisms (14). This plasmid encodes the LuxR regulatory protein from *V. fischeri*, which in turn controls expression of bioluminescence from the *Photorhabdus luminescens luxCDABE* operon present on the same plasmid. The presence of this system in *S. liquefaciens* monitors the presence of AHL in the host bacterium as well as monitoring autoinduction of AHL production. The latter is reflected in a sudden burst in the emission of bioluminescence once the required bacterial density has been reached (Fig. 6). If the *D. pulchra* compounds block AHL signalling, autoinduction of bioluminescence would be inhibited. As shown in Fig. 6, bioluminescence in growing cultures of *S. liquefaciens* harboring the monitor plasmid was inhibited by 100 μg of the two furanones per ml. Most importantly, at the transition between the exponential and stationary growth phases, the autoinduction of bioluminescence was clearly switched off by compound 1 and delayed by compound 2 in relation to bacterial density.

To confirm that the concentrations which inhibited AHL-induced bioluminescence did not interfere with physiological requirements for the light-emitting reaction, we tested if addition of the *D. pulchra* compounds interfered with expression of bioluminescence from *Escherichia coli* cells harboring a constitutively expressed *lux* fusion present on the plasmid pMRS15D (12). We showed that the concentrations found to switch off AHL-induced bioluminescence did not affect expression of bioluminescence in the constitutive system (data not shown).

We have also examined the effect of *D. pulchra* compounds on AHL-dependent phenotypic responses in marine bacteria. The expression of bioluminescence in *V. fischeri* and *V. harveyi* was reduced 50- to 100-fold in the presence of 10 to 20 μg of the *D. pulchra* compounds per ml (data not shown). For both of these bacteria, this compound-induced effect occurred at concentrations that did not reduce their rate of growth.

In this study, we have used *S. liquefaciens* or *E. coli* constructs as model organisms to investigate interference with AHL-driven systems by furanones, because the details of the AHL regulatory systems are well known for these organisms. These organisms do not encounter *D. pulchra* in the field. However, swarming in general is likely to play an important role in bacterial colonization of submerged surfaces (swarm cells have been observed in biofilms [20]), and we believe our results in general have considerable relevance both for the interaction between *D. pulchra* and associated bacteria and more broadly for chemically mediated interactions between eukaryotes and prokaryotes. This contention is based on two considerations. First, furanones also inhibit swarming motility of marine bacteria, including those isolated from *D. pulchra*. For example, Maximilien et al. (22) have shown that nearly 25% of 180 bacterial strains isolated from the surface of this alga or from the nearby habitats exhibit swarming motility. Six of these isolates (including one *Serratia* species) were examined in detail, and in swarming assays, furanones added at between 5 and 25 μg/ml of medium slowed or inhibited swarming in all six isolates without affecting growth (22). Furanones also inhibited attachment of these isolates at non-growth-inhibitory concentrations, both when surfaces were coated with the compounds and when cells were preincubated with furanones (22). Thus, furanones inhibit important surface colonization traits in ecologically relevant bacteria. Second, furanones inhibit these phenotypes at ecologically relevant concentrations. The concentrations of individual furanones found on the surface of *D. pulchra* in the field range between 10 ng and 5 $\mu g/cm^2$ (10), and these levels strongly inhibit bacterial attachment in both laboratory and field assays (22). The concentrations used in the swarming assays in this study are well within this range. A theoretical concentration of furanones on the surface of the plates can be calculated by assuming that swarming cells will only encounter furanones in the upper 10 μm of agar. Assuming homogeneity of furanones in the plates, then a concentration of furanones of 100 μg/ml translates to a "surface" concentration of 100 ng/cm^2—well within the range of actual concentrations on the plant.

Bacteria can be highly detrimental to marine algae and other eukaryotes (21). Moreover, because bacteria are generally the first colonizers of submerged surfaces, the abundance and composition of the bacterial community on the surface of an alga will significantly affect the subsequent development of a macrofouling community (18). Furanones from *D. pulchra* have strong inhibitory effects against bacteria, and thus these compounds may play an important role in algal-microbial interactions. Moreover, our evidence to date suggests that this inhibition may occur via interference with AHL regulatory systems. Thus, the ecological relevance of AHL regulatory systems may extend well beyond bacterium-bacterium interactions (19). Since the harmful activities of a number of plant and animal pathogens are regulated by AHL-driven systems (15, 24), the ability of furanones to inhibit AHL systems may also have important applied implications.

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