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REVIEW

Do marine natural products interfere with prokaryotic AHL regulatory systems?

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ABSTRACT: Recent studies indicate that a taxonomically diverse range of marine eukaryotes produce metabolites which inhibit phenotypic traits in bacteria, with no or minimal effects on growth. In this review, we present evidence for the existence of such eukaryotic interference with a conserved prokaryotic signalling system. We demonstrate that halogenated furanones, a class of secondary metabolites produced by the Australian subtidal red alga *Delisea pulchra*, interfere with the acylated homoserine lactone (AHL) regulatory system in several Gram-negative bacteria. Furanones were found to interfere with the AHL mediated expression of bioluminescence, swarming (surface) motility, and exoenzyme synthesis in different bacterial species. Furthermore, adhesion and swarming in a range of marine bacteria, for which the identity of the signalling molecules is not yet determined, were inhibited by furanones at concentrations that did not affect growth. Evidence for these effects were obtained in both field and laboratory experiments. Competition experiments in the presence of different concentrations of AHLs and furanones showed that the expression of swarming and bioluminescence in laboratory strains is competitively inhibited in a fashion that suggests that both classes of compounds have affinity for the same receptor site in the AHL regulatory system. Finally, by performing structure-function experiments on the inhibition of AHL systems by a range of different furanones, we identified the structural prerequisites responsible for interference.

KEY WORDS: AHL signalling · AHL antagonists · Furanones · Marine bacteria · *Delisea pulchra*

INTRODUCTION

Recent research into the means by which bacteria communicate and sense the environment has revealed the existence of an apparently widespread and conserved bacterial regulatory system, the acylated homoserine lactone (AHL) regulatory system (Salmond et al. 1995, Fuqua et al. 1996, Swift et al. 1996). Bacteria use this system for the expression of a large number of phenotypes, particularly those that facilitate their colonization on or in higher organisms. An increasing number of bacterial species, which display an increasing number of colonization related phenotypes, have been proven or suggested to employ the AHL regulatory system for the expression of phenotypes that aid in surface colonization and invasion of a variety of eukaryotic hosts (Passador et al. 1993, Pirhonen et al. 1993, Zhang et al. 1993, Eberl et al. 1996). Given that the expression of such traits allows for the effective establishment of a bacterial population on or in higher organisms, it has been hypothesized that the host may have developed specific means of interfering with the expression of the phenotypic traits that lead to the establishment of a host associated bacterial population. It has been proposed that the production of compounds, by higher organisms, which interfere with the mode of action of the homoserine lactone signalling

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molecules, will offer not only an efficient defense against the colonization and subsequent invasion by bacteria, but also a means by which the host can manipulate the extent and composition of the host associated bacterial population (Givskov et al. 1996). In this review, we explore eukaryotic interference with a conserved prokaryotic signalling system. The experimental model is based on the association between bacteria and the Australian subtidal red alga *Delisea pulchra*, which produces a range of low molecular weight molecules that are inhibitory against a range of organisms that normally form biofilm and biofouling communities in the marine system (de Nys et al. 1995). An extension of the study is that an understanding of interactions of signalling molecules produced by both the bacteria and their hosts is of considerable importance not only in ecology but also in a series of biological applications, in particular the prevention of bacterially induced disease.

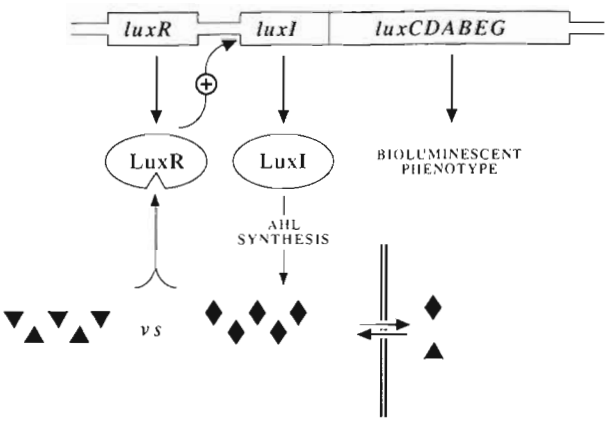


Fig. 1. Diagrammatic description of the inhibition of AHL mediated gene transcription by furanones of the alga *Delisea pulchra* as illustrated through the model bioluminescent *lux* system of *Vibrio fischeri*. Furanones (▲) are proposed to compete with AHLs (◆) for a binding site on the LuxR protein interfering with transcription of the *luxICDABEG* operon

CONSERVED BACTERIAL REGULATORY CIRCUIT: THE HOMOSERINE LACTONE MEDIATED SIGNALLING SYSTEM

AHL mediated gene expression is a conserved regulatory system, which is now well characterized in a broad range of Gram-negative bacteria. This system is traditionally considered to be a mechanism by which bacterial cells express genes in response to their population size. It involves the production of a small diffusible signal molecule, acyl homoserine lactone, which accumulates in the surrounding environment. Above a certain threshold concentration, this molecule binds to a regulatory protein (LuxR or a LuxR analogue) which directs the expression of relevant genes (Salmond et al. 1995, Fuqua et al. 1996, Swift et al. 1996).

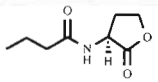
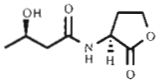
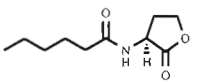
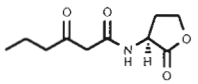
The best studied AHL regulatory system is that of the marine bacterial symbiont *Vibrio fischeri* (Fig. 1) (Meighen & Dunlap 1993). The model for this regulatory circuit describes the mechanism for population density-dependent gene activation by the LuxR-LuxI family of transcriptional regulators which are common in a diverse group of Gram-negative bacteria. In *V. fischeri* the phenotype of bioluminescence is controlled by the regulatory pair. Known as quorum sensing, self produced extracellular signal compounds (autoinducers) interact with transcriptional activator proteins. The *I* gene encodes a synthase that produces the AHL signalling molecule, the autoinducer. The autoinducer binds at a receptor site on the R protein which then becomes activated and serves as a transcriptional activator. The activated R protein serves to induce transcription of not only the structural genes but also the *I* gene, hence the autoinduction system.

While not further explored in this review, there are a number of variations in the organization of the AHL regulatory system in different bacteria (Fuqua et al. 1996). More complex systems are gradually being elucidated such as that of the AHL system of *Vibrio harveyi* (Bassler et al. 1994). Here, the autoinducer binds to the sensor (receiver) protein of a phospho-relay 2-component regulatory system, another conserved regulatory system. The binding of the signal leads to phosphorylation of a response regulator, which upon activation removes a DNA binding repressor protein and thereby allows for the R regulator to bind to the DNA and serve as a trancriptional activator.

The regulatory part of the AHL system, i.e. the R-I pair and autoinduction mechanism, is evolutionarily conserved in many bacteria; there exists today information on such systems, with modifications, in more than 15 bacterial species (Fuqua et al. 1996, Swift et al. 1996). The structural genes are different in different bacteria and constitute the genes that are needed for the appropriate phenotype to be expressed in individual bacterial species.

The list of AHLs that are involved as autoinducers in AHL mediated gene expression is growing. The structures that have been elucidated to date demonstrate variation in the length and the number and types of substitutions on the side chain (Table 1). For several of the AHL systems there is crosstalk, i.e. signals from another species will induce the AHL regulatory system normally driven by a specific AHL molecule, while in other cases, dependent on the configuration of the R receptor and the AHL species, there is no or very little crosstalk (Greenberg et al. 1979, Swift et al. 1996).

Table 1. Bacterial AHL-regulated phenotypes which are down regulated by furanones

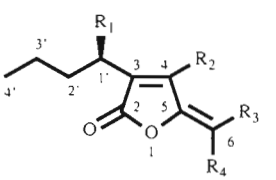
Chemical formula	Name	Organism	Phenotype
	*N*-butanoyl-L-homoserine lactone (BHL)	*Serratia liquefaciens*	Swarming (Giskov et al. 1996) Exoproteases (Gram, de Nys, Givskov, Steinberg & Kjelleberg unpubl.)
	*N*-(3-hydroxy)-butanoyl-L-homoserine lactone (HBHL)	*Vibrio harveyi*	Bioluminescence, exoproteases (Manefield, de Nys, Steinberg & Kjelleberg unpubl.)
	*N*-hexanoyl-L-homoserine lactone (HHL)	*Chromobacterium violaceum*	Pigment (authors' unpubl. data)
	*N*-(3-oxo)-hexanoyl-L-homoserine lactone (OHHL)	*Vibrio fischeri* *Erwinia carotovora*	Bioluminescence (Giskov et al. 1996) Exoenzymes (authors' unpubl. data)

Similarly, the list of phenotypes known to be controlled by LuxR-LuxI type regulatory systems is growing and includes a series of unrelated characteristics (Table 1). Furthermore, lectin production in *Pseudomonas aeruginosa* seems to be controlled by an AHL system (Swift et al. 1996) and we suggest in this review that signalling systems are involved in mediating adhesion of marine bacteria. Moreover, non-cell density traits such as cell division (Garcia-Lara et al. 1996, Sitnikov et al. 1996), adaptation to non-growth or stationary phase (Gray et al. 1996, S. Srinivasan, J. Ostling, T. Charlton, R. de Nys, K. Takayama & S. Kjelleberg unpubl.), and outgrowth of the starved cell (Cooper et al. 1995) should possibly be added to this list. Several such phenotypes can be accommodated by single cells and the case for regulation of bioluminescence by stationary phase regulations has been made (S. Ulitzur unpubl.). In addition AHL and AHL-like mediated entry into stationary phase and induction of starvation responders have been demonstrated on a *Vibrio* species and in *Rhizobium leguminosarum* at relatively low cell densities (Gray et al. 1996, Srinivasan et al. unpubl.). Evidence for induction of the stationary phase sigma factor RpoS by both acylated and non-acylated homoserine lactone has been presented (Huisman & Kolter 1994, Latifi et al. 1996).

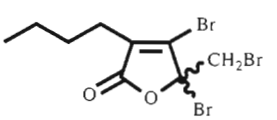
Interestingly, many of the phenotypes that have been reported to date point to a recurring theme of colonization. An important question therefore is how the willing or unwilling hosts have learnt to deal with or defend themselves against the colonization by bacterial strains that employ AHL systems. The question specifically addressed in this review is whether higher organisms produce compounds that prevent bacteria from using their signalling molecules, or whether they serve as intra- or extracellular signals in the bacterial colonizers.

HALOGENATED FURANONES PRODUCED BY THE RED ALGA *DELISEA PULCHRA*

Many marine higher organisms successfully defend themselves against fouling. For example, the Australian subtidal red alga *Delisea pulchra* inhibits fouling by the production of a group of secondary metabolites known as furanones (Kazlaukas et al. 1977, de Nys et al. 1993, 1995). These compounds vary in their substitutions on the side chain, the ring, and the exocyclic double bond (Fig. 2). They are stored in specialized vesicles and are released at the surface of the thallus (S. Dworjanyn, R. de Nys & P. Steinberg unpubl.). The furanones have been demonstrated to be effective in



Furanone	R1	R2	R3	R4
1	H	Br	Br	Br
2	H	Br	H	Br
3	OAc	Br	H	Br
4	OH	Br	H	Br
5	OAc	Br	H	I
8	H	H	Br	Br
10	OAc	Br	Br	Br



Furanone 9

Fig. 2. Structure of natural and synthetic furanones used in AHL regulation bioassay systems

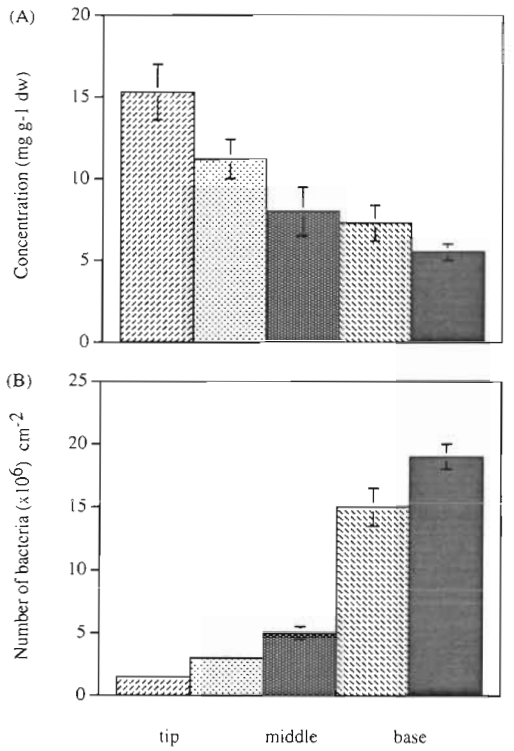


Fig. 3. (A) Concentration of total furanones in portions of *Delisea pulchra* from the tip of the plant to the base. Means ± SE, n = 10. (B) Abundance of bacteria on the surface of *D. pulchra* from the tip of the plant to the base. Means ± SE, n = 25. From de Nys et al. (1996) and Maximilien et al. (unpubl.)

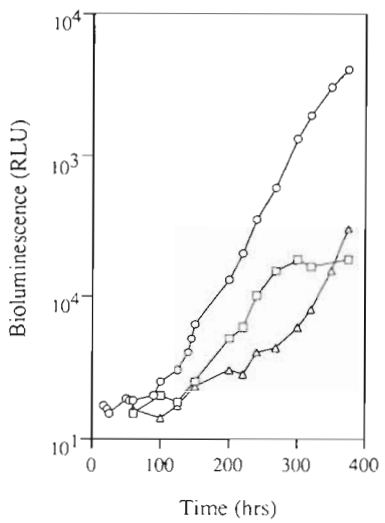


Fig. 4. *Serratia liquefaciens* cells harbouring the bioluminescent AHL monitor plasmid pSB403 were grown at 30°C in AB medium with 0.4% glucose and 0.5% Casamino acids for approximately 10 generations prior to splitting into 3 subcultures, 2 of which were supplemented with either *Delisea pulchra* compound 1 or 2 at 100 µg ml^{-1}. Expression of bioluminescence (RLU: relative light units) in the absence (○) or presence of *D. pulchra* compounds 1(□) and 2(△). Emission of bioluminescence was measured in a Turner TD-20e lumi-nometer (Turner designs, Sunnyvale, CA, USA). From Giskov et al. (1996)

preventing a series of common fouling organisms (de Nys et al. 1995).

More importantly, in the context of this review, furanones also prevent fouling by the primary colonizers, the marine bacteria, and hence the formation of a bacterial biofilm (R. Maximilien, R. de Nys, C. Holmstrom, L. Gram, M. Givskov, K. Crass, S. Kjelleberg & P. D. Steinberg unpubl.). Field data have demonstrated that the concentration of furanones is inversely correlated to the degree of bacterial colonization (de Nys et al. 1996, Maximilien et al. unpubl.) (Fig. 3). Furthermore, scanning electron microscopy of various parts of the plant surface shows that the sites of maximum production of the furanones are essentially free of bacteria.

The ability of furanones to prevent colonization of bacteria at the surface of the alga raises the question whether the furanones which are structurally similar to AHLs act as biomimics interfering with expression of AHL regulated phenotypes. This conceivably occurs by binding of the furanones to the receptor site of the R protein (Fig. 1), and, if correct, would be the first identification of AHL biomimics, produced by higher organisms.

INHIBITION OF AHL BIOASSAY SYSTEMS AND PHENOTYPES BY HALOGENATED FURANONES

To test the hypothesis of specific interference of AHL regulated phenotypes by furanones, we conducted a series of experiments using AHL bioassay systems. Three such systems were studied in detail: bioluminescence, swarming or surface motility, and exoenzyme production.

Bioluminescence, which was the first discovered AHL driven phenotype, and has been the principle model for studies of the AHL regulatory system, is easily quantifiable and lends itself to precise and conclusive experiments. We employed a monitoring system for high output of light, the plasmid pSB403 in *Serratia liquefaciens* and in *Escherichia coli* consisting of the cassette for the structural *lux* genes from *Photorhabdus luminescens* and the R protein from *Vibrio fischeri* (Eberl et al. 1996). This system responds to the concentration of autoinducer that can be recognized by the R protein. Addition of furanones resulted in a marked, specific, down regulation of bioluminescence in a fashion indicative of interference with the autoinduction circuit, at concentrations that do not affect growth (Fig. 4) (Givskov et al. 1996). In order to rule out that the reduction in light emission is not due to the inhibition of the *lux* genes or their products, or reflects a general effect that is manifested in the energy-dependent and sensitive expression of light, we tested the effect of addition of furanones to *E. coli* harbouring pMRS15D

which displays a constitutive expression of light from *luxAB*. Furanones showed no effect on the emission of light from this plasmid (Givskov et al. 1996). This result, in concert with the absence of effect of the furanones on the swimming motility and growth of *S. liquefaciens* (Givskov et al. 1996), indicates that the furanones target the AHL system.

Swarming motility is a characteristic of great ecological relevance (Belas 1992, Harshey 1994). In *Serratia liquefaciens* it is a readily observable AHL regulated phenotype (Eberl et al. 1996). Surface motility by means of swarming allows bacteria to rapidly colonize a surface and they do so as a result of a complex differentiation process which leads to elongated and hyperflagellated swarmer cells that move in packs. In *S. liquefaciens* it is known that swarming is mediated by the diffusable signal molecule *N*-butanoyl-L-homoserine lactone (BHL) which is proposed to bind to the SwrR regulatory protein. When activated, SwrR is believed to operate as a transcriptional activator, analogous to the LuxR regulator (Eberl et al. 1996). Swarming on a plate, which is the bioassay we employed in these experiments, can easily and accurately be followed over time, making it a quantifiable bioassay. At the edge of the swarming colony, the elongated cells move in a coordinated fashion and this allows for rapid spreading of the population. The effect of furanones on swarming motility is demonstrated in Fig. 5. Increasing concentrations of furanone 2 progressively reduces the speed by which the swarming colony expands.

Exoenzymes are often employed by bacteria in the processes of colonization and invasion of higher organisms (Atlas & Bartha 1993), and are regulated by the AHL system in several bacterial species (Swift et al. 1996). We have added to the suit of exoenzymes known to be controlled by AHLs, with the discovery of stationary phase exoproteases produced by *Serratia liquefaciens* (Eberl et al. 1996). Addition of furanones to *S. liquefaciens* significantly reduces the total exoprotease activity, without any effects on growth (L. Gram, R. de Nys, M. Givskov, P. D. Steinberg & S. Kjelleberg unpubl.). Subsequent experiments revealed that this shut down in overall exoprotease activity is due to the specific down regulation of 2 proteins of 55 and 51 kDa in size (Gram et al. unpubl.). The experiments performed in this system included the down regulation of the 2 exoproteases in the *S. liquefaciens* wild-type strain, the demonstration that these 2 exoproteins are down regulated in the *swrI*$^-$ mutant employed, and that the addition of BHL, the major autoinducer species in *S. liquefaciens*, to the *swrI*$^-$ mutant restores the expression of the 2 exoproteases to wild-type levels. We have recently also performed a series of experiments on the marine bacterial shellfish pathogen *Vibrio harveyi* and identified and down regulated, by the addition of furanones, 2 novel AHL regulated exoproteins that are determinants for the virulence by *V. harveyi* in prawns (L. Harris, M. Manefield, R. de Nys, P. D. Steinberg & S. Kjelleberg unpubl.).

In addition to the AHL regulated phenotypes presented above, we have explored other AHL mediated phenotypes, in different bacterial species, and have provided further evidence for the down regulation of specific AHL regulated traits by the addition of furanones. In summary, we have demonstrated that furanones interfere, in a specific and non-growth inhibitory fashion, with the following AHL regulated bacterial phenotypes: swarming (*Serratia liquefaciens*) (Givskov et al. 1996), exoenzyme production (*S. liquefaciens*, *Vibrio harveyi*, *Erwinia carotovora*, *Pseudomonas aeruginosa*) (Gram et al. unpubl., authors' unpubl. data, Harris et al. unpubl.), bioluminescence (*V. fischeri*, *V. harveyi*) (Givskov et al. 1996, Harris et al. unpubl.), and pigment production (*Chromobacterium violaceum*) (authors' unpubl. data). Recently we have shown that furanones induce abnormal, uncoordinated swarming motility in *Proteus mirabilis* (Gram et al. 1996).

COMPETITION BETWEEN AHLS AND FURANONES AND STRUCTURE-FUNCTION ANALYSIS

While the bioassay experiments are strongly supportive of an AHL biomimicry action by the furanones, the site of action of the furanone species remains to be conclusively demonstrated. To begin to investigate this, we have conducted a series of competition experiments for the expression of bioluminescence and swarming motility. It is clear that furanone species cannot interfere with or bind to the synthase protein (I protein), given that the monitor system we employed in the bioluminescence bioassay experiments does not have such a gene (Givskov et al. 1996). In the competitive binding experiments, increasing concentrations of AHLs were added in the presence of a furanone at a concentration that resulted in significant inhibition of the phenotype (Givskov et al. 1996, M. Manefield, L. Gram, L. Harris, R. de Nys, M. Givskov, P. D. Steinberg & S. Kjelleberg unpubl.). The addition of AHLs restored the phenotype. The extent of restoration depended on the concentration of the 2 competing signals reflecting the affinity of the AHL and different furanones for the common receptor site. To conclusively prove this hypothesis, binding experiments showing the displacement of labelled AHLs with furanones in LuxR and SwrR overproducing systems are currently being performed.

These results also demonstrate that it is possible to perform structure-function experiments, on the AHL system of interest, and thereby identify the structural

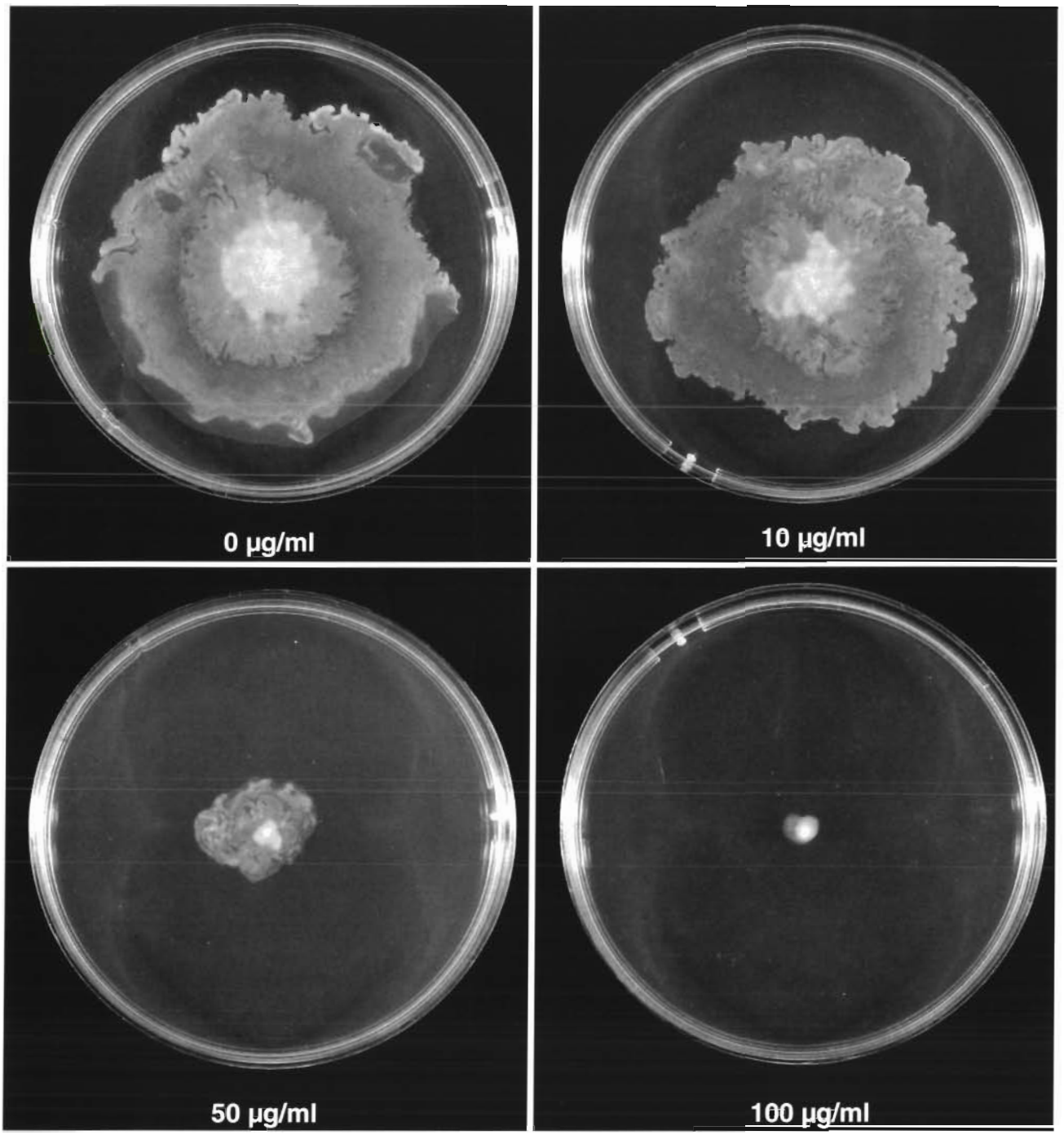


Fig. 5. Effect of increasing concentrations (0, 10, 50, 100 µg ml^{-1}) of *Delisea pulchra* furanone 2 on *Serratia liquefaciens* swarming motility. Agar plates were stab inoculated at the center from an exponentially growing culture (OD_{450} of approximately 0.5) and incubated at 30°C. Colonies photographed 20 h after inoculation. From Givskov et al. (1996)

prerequisites and moieties responsible for interference. These experiments are similar to those recently reported for a range of AHL molecules in binding to the *Vibrio fischeri* LuxR receptor (Eberhard et al. 1986, Schaefer et al. 1996) as well as the *Erwinia carotovora* LuxR analogue CarR (Chhabra et al. 1993). We have performed detailed structure-function experiments for swarming motility in *Serratia liquefaciens* and bioluminescence, as regulated by the *V. fischeri* R protein (Manefield et al. unpubl.). Ranking the furanones ac-

cording to the extent of inhibition of the expression of the phenotype leads to specific information on structural constraints and dictates the synthesis of biomimics with desired properties. These results reveal the relative importance to inhibitory activity of the 5 membered ring, the presence of the exocyclic double bond at the carbon 5 position as well as an acetyl or hydroxyl group at the carbon 1' position. These structure-function experiments are most useful for pursuing more detailed experiments on the role of signalling communication between bacteria and higher organisms. The information provided based on structure-function experiments has significant benefits for a series of biomedical and agricultural applications in which AHL biomimics can be used to manipulate the expression of specific phenotypic traits.

FURANONES DOWN REGULATE SWARMING AND ADHESION BY MARINE BACTERIA

This review has described the interference by furanones with AHLs in a range of defined laboratory systems. What are the effects of such interference on colonization relevant phenotypes in marine bacteria from the surface of *Delisea pulchra* and other sites in marine waters? By coating surfaces with furanones at different concentrations and testing adhesion of bacteria to such surfaces in the field, the colonization or attachment is prevented at concentrations that occur naturally on the surface of the plant (Maximilien et al. unpubl.). In laboratory experiments adhesion of individual marine bacterial strains was significantly inhibited by naturally occurring concentrations of furanones (Fig. 6) (Maximilien et al. unpubl). The furanones were coated on test surfaces and were exposed to different bacterial strains, at different cell concentrations. However, the model for interference by furanones with bacterial signalling systems proposed in this review (also in Givskov et al. 1996) predicts that furanones interfere intracellularly rather than affecting substratum characteristics. To test this, cells of the marine bacterial isolate V36, isolated from the surface of a *Delisea pulchra*, were pre-incubated with and without furanones. The cells were washed and the degree of adhesion to control surfaces without furanones was measured (Maximilien et al. unpubl.). This caused a significant decrease in adhesion in V36 cells pre-incubated with furanones. Supporting the notion that adhesion is regulated by a signalling mediated system, adhesion of V36 was significantly higher following pre-incubation with the non-polar extract of the supernatant from stationary phase V36 cells. In addition to studies of the interference with bacterial adhesion, swarming motility in individual marine bacterial isolates was specifically down regulated by furanones, without any effect on growth (Maximilien et al. unpubl.). More than 30% of a collection of more than 100 bacterial isolates from different substrata in marine waters exhibited this type of surface motility.

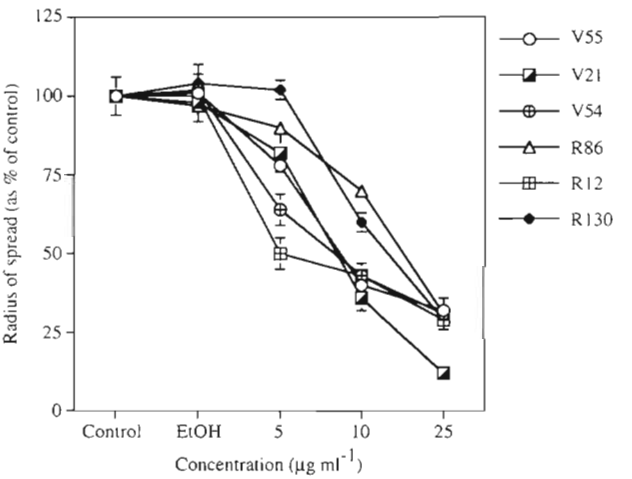


Fig. 6. Effect of furanone 2 on swarming motility in 6 marine bacterial strains. Means ± SE, n = 4. From Maximilien et al. (unpubl.)

ECOLOGICAL IMPLICATIONS

Bacteria have important and widespread effects on marine eukaryotes in natural ecosystems (Littler & Littler 1995, Kushmaro et al. 1996). We predict that eukaryote hosts have evolved defensive mechanisms against bacteria, and indeed, chemical defense against bacteria appears to be widespread among marine eukaryotes (reviews by Davis et al. 1989, Wahl 1989, 1996). This chemical inhibition of bacteria by potential hosts can take a number of forms, the simplest of which is the production of toxic metabolites which kill all bacteria. However, simple broad spectrum toxicity is not the only, and perhaps not even the most important, mechanism by which eukaryotes inhibit bacteria. Recent studies clearly indicate that a taxonomically diverse range of marine eukaryotes produce metabolites which inhibit specific bacterial properties such as those mediating adhesion (Wahl et al. 1994, Slattery et al. 1995, Maximilien et al. unpubl.). Such specific effects are likely to be advantageous to the host, since production of toxic metabolites means that the producing organism must cope with the autotoxic effects of those metabolites. Moreover, in some cases specific strains of bacteria may actually be beneficial to the host, as is the case for bacteria on the eggs of the shrimp *Palaeman macrodactylus* (e.g. Gil-Turnes et al. 1989), and as is implied by the common observation

that axenic culturing of marine eukaryotes is either impossible, or results in abnormal morphologies for the cultured organism. Thus the ability to interfere with specific bacterial properties may result in the colonization of the host by benign or positive strains, but not deleterious ones. This hypothesis is supported by observations on the differential effects of *Delisea* metabolites on host versus non-host strains of marine bacteria (Maximilien et al. unpubl).

Kell et al. (1995) have recently discussed the possibility that AHL regulated systems play an important role in ecological interactions between bacteria. Based on the effects of furanones on AHL regulated processes, and on the emerging evidence for specific and selective effects of eukaryote metabolites on bacterial properties, we suggest that eukaryotic interference with AHL regulated systems, and with bacterial signalling systems more generally, may also be common. Such interference raises the possibility of specific and complex chemical regulation of host-bacterial interactions.

CONCLUSIONS

Based on the findings presented in this review, it is proposed that:

- biomimics of AHLs are produced by higher organisms;
- such molecules specifically down regulate AHL mediated phenotypes in a wide range of bacteria;
- there is a high degree of specificity in the binding of the AHL biomimicry molecules, similar to the variation exhibited by different AHLs;
- adhesion as well as swarming of marine bacterial isolates are prevented by naturally occurring concentrations of furanones;
- furanones in the field prevent adhesion of bacteria to the host surface.

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