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Inhibitory Effects of Secondary Metabolites from the Red Alga *Delisea pulchra* on Swarming Motility of *Proteus mirabilis*

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Abnormal, uncoordinated swarming motility of the opportunistic human pathogen *Proteus mirabilis* was seen when a crude extract of the Australian red alga *Delisea pulchra* was added to the medium. This occurred at concentrations at which growth rate, swimming motility, cell elongation, polynucleation, and hyperflagellation were not affected. One halogenated furanone from *D. pulchra* inhibited swarming motility at concentrations that did not affect growth rate and swimming motility. Other structurally similar *D. pulchra* furanones had no effect on swarming, suggesting considerable specificity in the effects of furanones on swarming motility by *P. mirabilis*.

The swarming behavior of the opportunistic human pathogenic bacterium *Proteus mirabilis* enables it to colonize a variety of surfaces, including urinary catheters in hospitalized patients. This is a major cause of *P. mirabilis*-related urinary tract infections (1). The ability of the organism to invade urothelial cells and the expression of virulence factors, such as the production of exoenzymes, are also coupled to swarm cell formation (1–3). Swarm cell formation occurs in several stages (4, 6, 15). The first stage involves sensing cues in the environment. This is followed by a differentiation process in which the cells elongate, multinucleate, and develop extensive flagellation. Finally, the cells move rapidly across the surface in an aligned and highly coordinated way.

In other bacteria, the formation of a swarming colony follows essentially the same sequential pattern, including a differentiation similar to that of *P. mirabilis*. The formation of a swarming colony by *Serratia liquefaciens* involves cell-cell signalling by a quorum-sensing mechanism based on *N*-acyl-homoserine lactones (AHLs) (9). It was recently demonstrated that swarm cell differentiation and swarming motility of *S. liquefaciens* are inhibited by secondary metabolites produced by the Australian red alga *Delisea pulchra* (10). The *Delisea* compounds are brominated furanones (8) that show structural similarity with the AHL signal molecules and that specifically interfere with AHL-driven swarming in *S. liquefaciens* (10). The inhibitory effect exerted by these metabolites is not limited to *S. liquefaciens*, since the swarming motility of several marine bacterial isolates is also inhibited by the furanones (12a).

On the basis of this inhibition of bacterial swarming by *Delisea* furanones, we examined how furanones affect the process of surface colonization in *P. mirabilis*.

***D. pulchra* crude extract and pure furanone compounds.** Crude extracts were prepared from a dichloromethane extraction of freeze-dried *D. pulchra*. Furanones 1 to 4 (Fig. 1) were isolated by vacuum liquid chromatography of the crude extract followed by high-performance liquid chromatography as previously described by de Nys et al. (8).

***P. mirabilis* crude extract.** *P. mirabilis* was cultured in Luria broth 10 (LB10) with vigorous aeration at 25°C, and the cells were harvested when they reached an A_{450} of 3.0. A crude extract was prepared by dichloromethane extraction of the sterile filtered *P. mirabilis* supernatant.

Growth, swarming, and swimming conditions. *P. mirabilis* (UNSW 059300) was grown in LB10 (order no. 0446-17-3; Difco) in all experiments. Growth was monitored by measuring the A_{450} in liquid medium. LB10 plates for assays of swimming and swarming motility contained 0.3 and 1.5% Bacto Agar (Difco), respectively. The *D. pulchra* furanones were dissolved in 96% ethanol and added to growth media and LB10 plates (before solidification) in concentrations of 5, 10, 20, 40, and 100 μg ml^{-1}. Ethanol in appropriate amounts was added to control plates. The *P. mirabilis* supernatant extract was dissolved in 96% ethanol and added to the molten LB10 agar in concentrations of 1, 5, and 40 μg ml^{-1}. Agar plates were allowed to dry for a few hours before being stab inoculated with a *P. mirabilis* preculture grown in LB10 at 37°C. All plates and cultures were incubated at 37°C, and all experiments were done in duplicate. The distance from the point of inoculation to the rim of the swimming or swarming colony was measured at hourly intervals. The data are shown (see Fig. 3a and b) as means ± standard errors of the distances spread as a function of time. Cells at the front of the swimming or swarming colony were observed under ×400 magnification with a Leitz Vario Orthomat microscope with phase contrast. Nucleation was assessed by removing cells from the rim of the colony and adding 1 drop of 10 μg of 4′,6 diamidino-2-phenylindole (DAPI; order no. D9542; Sigma) ml^{-1}. The cells were immediately examined with an Olympus BH2 microscope equipped with epiflourescence optics. The excitation wavelength used was 350 nm, and the emission wavelength used was 450 nm.

Transmission electron microscopy. Cells were removed from LB10 plates (1.5% agar) by washing off the outer 5 mm of each colony. The washed cells were then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 30 min and finally washed three times in sterile MilliQ water. For negative staining, 1 drop of cell suspension was mixed with 1

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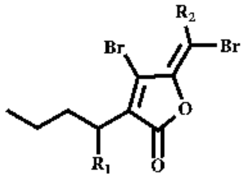

Furanone 1-4

	R1	R2
1	H	Br
2	H	H
3	OAc	H
4	OH	H

FIG. 1. Structures of furanones 1 to 4 from *D. pulchra.*

drop of sodium phosphotungstate stain (2% aqueous) for 10 s with a Formvar-coated grid (300 copper square grid). The grid was blotted with filter paper, air dried for 10 min, and examined immediately with a Hitachi H7000 electron microscope.

Effect of *D. pulchra* crude extract on behavior of *P. mirabilis*. On 1.5% agar plates, *P. mirabilis* exhibited normal swarming behavior in which bundles of elongated, hyperflagellated cells spread rapidly across the surface in a highly coordinated manner (Fig. 2a), interrupted by periods of consolidation. When crude extract from *D. pulchra* was added to the medium at 20 to 40 $\mu g\ ml^{-1}$, the distance traveled by the expanding colony was not different from that of the control (Fig. 3a) but the normal consolidation was not observed. Microscopic inspection revealed that the cells at the rim of the spreading colony were elongated (5 to 60 μm), motile, hyperflagellated (Fig. 4), and polynucleoid as assessed by DAPI staining (not shown). However, the cells were devoid of coordinated behavior (Fig.

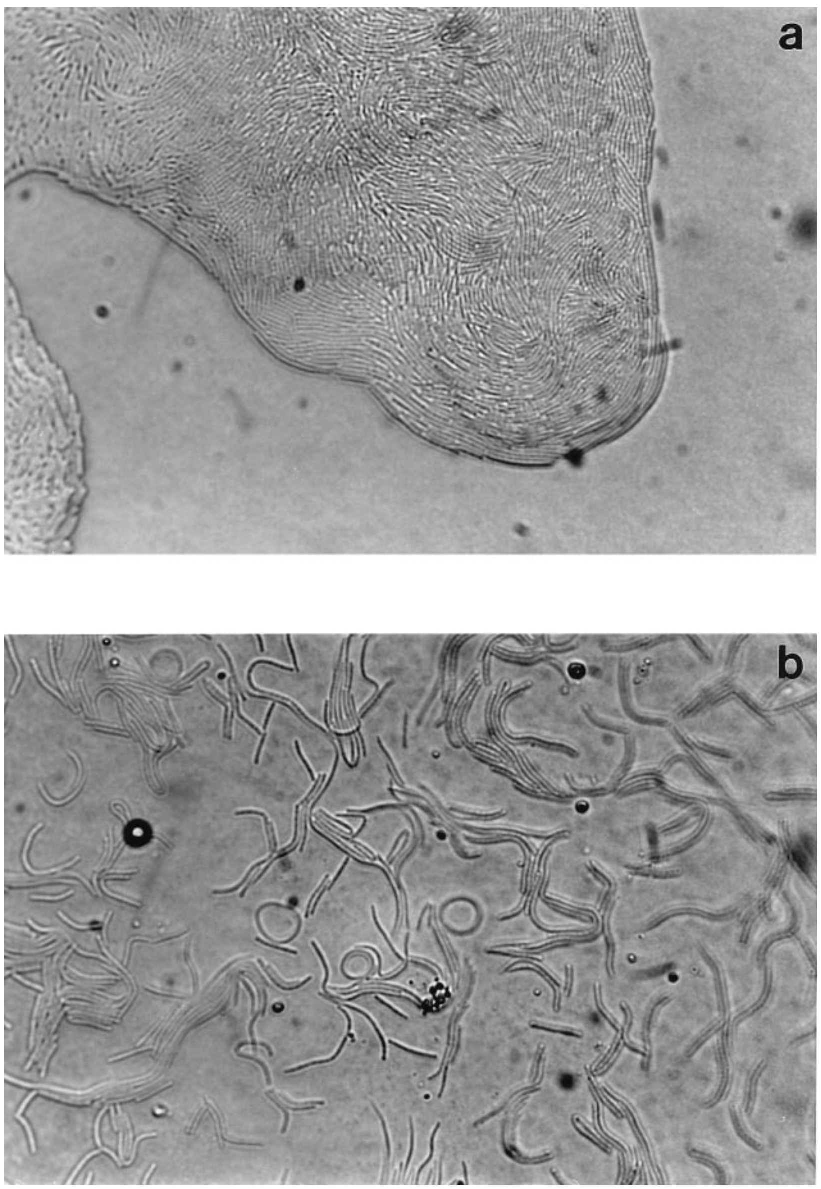

FIG. 2. Cells from the fronts of the spreading colonies of *P. mirabilis* on LB10 plates with 1.5% agar without (a) and with (b) *D. pulchra* crude extract (20 $\mu g\ ml^{-1}$). Plates were incubated at 37°C.

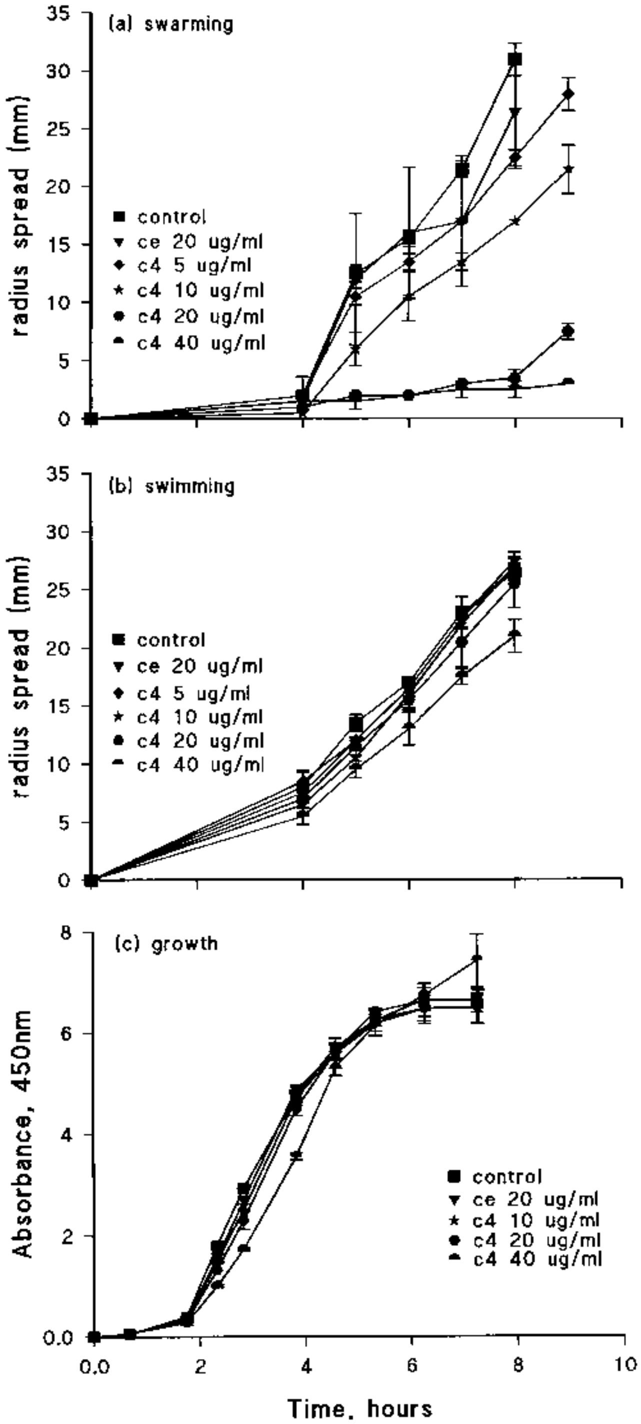

FIG. 3. Effects of *D. pulchra* crude extract (ce) and compound 4 (c4) on swarming motility (a), swimming motility (b), and growth (c) of *P. mirabilis* at 37°C. LB10 with 1.5% agar (a) and 0.3% agar (b) was used.

2b). Neither swimming motility as determined by the outward movement of concentric rings in 0.3% agar nor growth rate was affected (Fig. 3b and c). The crude extract was slightly growth inhibitory at concentrations above 50 μg ml^{-1} (data not shown).

Mutagenesis studies have indicated that the inhibition of swarming through the impairment of swarm cell formation or

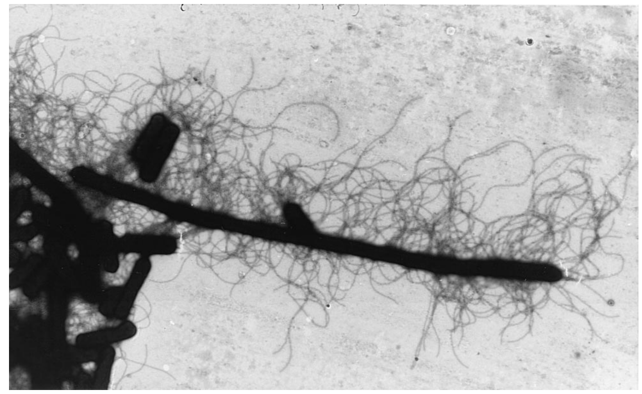

FIG. 4. Transmission electron microscopy of *P. mirabilis* cells grown on LB10 plates (1.5% agar) with 20 μg of *D. pulchra* crude extract ml^{-1}. See the text for details.

the crippling of swarming motility may occur at different points in cell differentiation (5, 13). Neither swarm cell formation nor motility was affected by the *D. pulchra* crude extract (Fig. 2 to 4), as the cells were elongated, hyperflagellated, and motile. Harshey (11) noted that isolated swarm cells rarely moved, suggesting that maintenance of close cell contact is essential (12). Exposure of *P. mirabilis* to the *D. pulchra* crude extract abolished close cell contact, but the cells remained highly motile and the colony spread to the same extent as a colony of swarm cells without the addition of crude extract.

Belas et al. (5) examined a number of swarming mutants of *P. mirabilis* designated crippled in swarming behavior. One mutant (BB2029) exhibited an uncoordinated migration resulting in an indeterminate consolidation pattern. The complete lack of cell alignment when *P. mirabilis* is exposed to crude extract from *D. pulchra* resembles the migration pattern of this particular mutant (5) and those of the Pat (pattern) mutants of *Serratia marcescens* 274 described by O'Rear et al. (13). The uncoordinated behavior could also resemble the phenomenon of sluggish movement in all directions of single cells, as is observed in the early stages of swarm colony formation (7).

Effects of *D. pulchra* furanones 1 to 4 on behavior of *P. mirabilis*. To assess the roles of furanone compounds in these phenomena, the four major furanones from *D. pulchra* (Fig. 1) were tested for their effects on swarming and swimming motility. The addition of pure compound 4 decreased the swarming velocity at a concentration of 10 μg ml^{-1} and significantly delayed the onset of swarming at a concentration of 20 μg ml^{-1} without affecting growth or swimming motility (Fig. 3). Swarming behavior was absent or dramatically delayed at 40 μg ml^{-1} (Fig. 3a), a concentration which had only a minor effect on growth (Fig. 3c) and did not affect swimming motility (Fig. 3b). Compounds 1 to 3 had no effect on swarming motility at 50 μg ml^{-1}, the highest concentration tested (data not shown).

The inhibition of swarming by furanones is similar to that observed in *S. liquefaciens* (10) and several marine bacteria (12a). However, none of the four pure furanones tested caused an effect similar to that of the crude extract. The difference in responses to crude extract and pure furanones may be due to a low concentration of the individual furanones in the crude extract. However, low concentrations of compound 4 did not cause the same effect as that of the crude extract. Alternatively, the uncoordinated behavior observed with crude extract may be due to interactive effects between compound 4 and other secondary metabolites.

Competition between *D. pulchra* furanones and *P. mirabilis* signal molecules? The mechanisms by which the *D. pulchra* furanones inhibit swarming are not known. The possible ability of *P. mirabilis* compounds to counteract the inhibitory effect of furanones was investigated by adding a crude extract of *P. mirabilis* supernatant to the swarming plates. As AHLs are involved in the swarming motility of *S. liquefaciens*, the counteracting effect of *N*-(butanoyl)-L-homoserine lactone (BHL) was also investigated. Neither the *P. mirabilis* extract nor BHL was able to overcome the inhibitory effect of compound 4.

Several of the swarming mutants examined by Belas et al. (5) were suggested to be defective either in synthesizing or in sensing the signals which are assumed to coordinate the processes of swarmer cell differentiation and consolidation. Such signals have not yet been described for *P. mirabilis*; however, the formation of a swarming colony of *S. liquefaciens* involves a quorum-sensing mechanism relying on AHL signal molecules that are released into the growth medium (9). Swarming motility in *S. liquefaciens* is inhibited in the presence of furanone compounds from *D. pulchra* (10). Two of these furanones interacted with an AHL-controlled reporter system, suggesting that their effect on swarming motility is due to suppression of the AHL autoinduction circuit (10). Several members of the family *Enterobacteriaceae* produce AHLs (14), but they have not to our knowledge been demonstrated in *P. mirabilis*. If swarming motility in *P. mirabilis* is dependent on a similar signalling circuit, it is speculated that the furanones specifically interfere with this system. The results presented here are consistent with the involvement of such a system, given the specificity of action of the furanones on swarming motility. Addition of a cell-free *Proteus* supernatant extract or pure BHL to the swarming plates did not overcome the inhibition caused by compound 4, and therefore a mechanism of competitive inhibition could not be demonstrated. However, in similar experiments done with an AHL-negative mutant of *S. liquefaciens*, we were able to demonstrate competition between furanone compounds and BHL (10). Competition could not convincingly be shown in the *S. liquefaciens* wild type.

Conclusions. The ability of *P. mirabilis* to differentiate into swarmer cells plays an important role in pathogenicity (1) as well as interferes with the diagnostic assessment of bacterial pathogens. As a result, many attempts have been made to find growth conditions that block swarming motility. Compounds used so far either affect growth (e.g., antibiotics), development of flagellation, or swarm cell differentiation (2, 15). The ability of furanones from *D. pulchra* to specifically block swarm cell differentiation therefore has obvious applications.

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