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Settlement behavior and metamorphosis of oyster larvae (*Crassostrea gigas*) in response to bacterial supernatants*, **

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Abstract. Veliger larvae of the oyster *Crassostrea gigas* (Thunberg) responded to unknown dissolved chemical inducers found in supernatants of cultures of the bacteria *Alteromonas colwelliana* and *Vibrio cholerae*. The response, which was similar to that seen when larvae were exposed to the neurotransmitter precursor L-3,4-dihydroxyphenylalanine (L-DOPA), consisted of an initial settlement phase of swimming with the foot extended and crawling on the substrate. Subsequently larvae attached to the substrate and metamorphosed. The percentage of veligers metamorphosing following induction of settlement behavior was higher in a group of older larvae, a response similar to that seen with L-DOPA, suggesting that competence to respond to bacterial supernatants is divided into two phases: behavioral competence followed by morphogenetic competence. Following size exclusion chromatography, the molecular weight of the peak containing the activity which induced settlement behavior was determined to be ≤ 300 daltons. Autoclaved Marine Broth, which induced low levels of settlement behavior also contained this low molecular weight active peak, suggesting that an oyster settlement inducer is also present in this medium.

Introduction

Many sessile invertebrates, including *Crassostrea gigas*, have larvae that are free-swimming until they are ready, or competent, to metamorphose. Metamorphic competence is thought to reflect the completion of development of an intact biochemical and neuronal pathway enabling the larva to perceive and respond to external cues that indicate an appropriate substrate for settlement and metamorphosis (Pechenik 1984, Hadfield 1986, Coon et al. 1990). The first step of this pathway is perception of an environmental cue(s) often including a chemical factor, and is thought to be mediated by membrane receptors (Burke 1983, Hadfield 1984, Morse 1985). Trapido-Rosenthal and Morse (1985, 1986) have presented evidence that receptors in 2-d old precompetent abalone larvae are habituated when exposed to chemical inducers, suggesting that while the receptors are present, the effector system (perhaps including neuronal components) is not functional. Similarly, precompetent larvae of the nudibranch *Phestilla sibogae* exposed to natural inducer substances fail to metamorphose when they would normally become competent, although this habituation can be reversed upon exposure of the larvae to clean seawater (Hadfield and Scheuer 1985, Hirata and Hadfield 1986).

In oysters, metamorphic competence has been divided into two stages: (1) behavioral competence, involving acquisition of the ability to respond to appropriate environmental stimuli with stereotypical settlement behavior including extension of the foot while swimming, then crawling; and (2) morphogenetic competence involving acquisition of the ability to respond to endogenous signals with morphological and physiological metamorphosis (Coon et al. 1985, 1990). Veligers of the Pacific oyster *Crassostrea gigas* have been shown to attain behavioral competence 2 to 4 d before attaining morphogenetic competence in laboratory experiments, suggesting that potential membrane receptors and/or internal pathways responsible for triggering the settlement behavior are intact before the morphogenetic pathways are functional (Coon et al. 1990). These experiments, however, used the neurotransmitter precursor L-3,4-dihydroxyphenylalanine (L-DOPA) to trigger settlement behavior, and used epinephrine (EPI) to trigger metamorphosis without prior settlement behavior (Coon et al. 1985), short-circuiting the putative external receptors that may respond to a variety of environmental stimuli, including natural inducing chemicals (Coon et al. 1990).

The involvement of bacteria in natural induction of settlement and metamorphosis in marine invertebrate larvae has been known for many years (see Bonar et al.

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1986 for review), and includes both the bacterial films on surfaces and soluble cues. The formation of pioneer microbial communities on surfaces appears to be beneficial to attachment and subsequent development of many marine larvae (Meadows and Campbell 1972, Scheltema 1974), including oyster pediveligers (Weiner et al. 1985, Walch et al. 1987). Whether larvae require contact with such films, or may sense soluble cues emanating from the film, has not been determined for most bacterial species. Dissolved bacterial inducers have been implicated in the metamorphosis of larvae of some invertebrates, including larvae of the polychaete *Janua brasiliensis* (Kirchman et al. 1982), planulae of the scyphozoan *Cassiopea andromeda* (Neumann 1979, Hofmann and Brand 1987), and planulae of the hydroid *Hydractinia echinata* (Müller 1973).

In this paper we describe for the first time the ability of oyster larvae both to initiate settlement behavior and to complete metamorphosis in response to a variety of bacterial supernatants. We also show that the percentage of veligers metamorphosing subsequent to behavioral induction with bacterial supernatants increases as the veligers age, and do so in a fashion similar to that seen with L-DOPA induction (see Coon et al. 1990). In addition, we show that oyster larvae exhibit low levels of settlement behavior when exposed to autoclaved Marine Broth containing no bacteria, suggesting that an inducer is present in this medium.

Materials and methods

Experimental oysters

Larvae of the Pacific oyster *Crassostrea gigas* (Thunberg) were obtained from Coast Oyster Hatchery of Quilcene, Washington and maintained in the laboratory as described in Coon et al. (1990). The larvae used in the experiments described below were from Groups B and C3 used by Coon et al. (1990). Group B larvae (shell length = 312 ± 11.2 μm, 21-d-old and 55% competent to settle and metamorphose upon arrival) were used for all experiments except those which tested for effects of larval age, in which larvae 5-d younger than Group B were used for comparison (Group C3: shell length = 290 ± 14.2 μm, 16-d-old and 15% competent to settle and metamorphose upon arrival). All experiments reported here were conducted 11 to 12 d after arrival of the larvae at the University of Maryland (Days 10 and 11 of Expt II in Coon et al. 1990).

Bacterial supernatants

The bacterial species *Alteromonas colwelliana* (Strain "LST-D", Weiner et al. 1985, 1988), *Vibrio cholerae* 596-B and *V. cholerae* HTX (a pigmented mutant of *V. cholerae* 596-B) were grown in Marine Broth 2216 (MB) (Difco) and/or AG-medium (Instant Ocean containing aspartic acid 8.32 g l^{-1}, glutamic acid 9.2 g l^{-1}, yeast extract 1.0 g l^{-1}) on a shaker table at 25 °C. Samples of bacterial cultures were taken at various times during the growth cycle and centrifuged ($16\,000 \times g$) for 10 min at 4 °C). The supernatant was either used immediately in bioassays (see below) or lyophilized for later use. Solutions of autoclaved MB and AG medium containing no bacteria, and filtered seawater (0.45 μm) served as experimental controls.

Chromatography

Samples (10 ml) of supernatant were lyophilized, reconstituted in 2 ml of distilled water and loaded on a Sephadex G-10 size exclusion column [2.5 × 50 cm; exclusion limit molecular weight (MW) > 700 daltons]. The supernatant was eluted with distilled water and monitored using UV absorbance at 280 nm. All fractions from the separation were lyophilized, reconstituted in filtered (0.45 μm) seawater, and tested at concentrations ranging from 0 to 10 mg ml^{-1} for their ability to elicit veliger settlement behavior and metamorphosis as described below. Molecular weight of the active peak was estimated from elution profiles of azide (MW = 65 daltons), cobalt (MW = 129 daltons), reduced glutathione (MW = 300 daltons), and blue dextran (MW > 2 000 000 dalton in exclusion volume) in an elution solution of either distilled water or 0.15 *M* NaCl in 50 m*M* Tris buffer (pH 7.0).

Bioassay

Experiments were conducted in polystyrene 24-well tissue culture plates (Costar #3424) with 0.45 μm filtered seawater containing antibiotics [antibiotic saline (ABS); 100 μg ml^{-1} each of penicillin-G, streptomycin sulfate, neomycin sulfate), in which 20 to 30 veligers were placed. Veliger settlement behavior in experimental solutions was monitored for 1 min every 10 min for 1 h as described in Coon et al. (1990). In order to determine the ability of veligers to metamorphose following exposure to bacterial supernatant, veligers were removed from supernatants after various exposure times, rinsed, and placed in fresh ABS. The number of veligers subsequently metamorphosing was determined 2 to 5 d later by counting the oysters showing shell growth and gill development.

Two replicates of each treatment were monitored in each experiment, except where noted. Actual data means are given in the figures and the table. Replicates were typically within 15% of the mean. Data were arcsin transformed for all statistical tests, which were considered significant when $p < 0.05$.

Results

Effects of supernatant age and concentration

Supernatants from bacterial cultures 0 to 6-d-old were tested at concentrations of 0 (seawater control), 10, 25, 50 and 100% supernatant (diluted with seawater) for their ability to elicit settlement behavior and metamorphosis of veligers (Figs. 1, 2, 3). In most cases the inductive activity of the supernatants increased with the age of the culture up to 50 h, but in some cases larval responses were reduced in high concentrations of supernatant and in old cultures. When *Alteromonas colwelliana* was grown in AG medium, both larval settlement behavior and metamorphosis increased with increasing supernatant concentration, peaking at 60 to 80% response (Fig. 1). When *A. colwelliana* and *Vibrio cholerae* were grown in MB, however, supernatants from the final two sampling times induced significantly less settlement behavior at 100% strength than at 50% dilution (Figs. 2A and 3A, B; $p < 0.05$, paired *t*-tests comparing 100% strength to 50% dilution), but did not have a significant negative effect on the number of larvae induced to subsequently metamorphose (Figs. 2B and 3C, D; $p > 0.2$, paired *t*-test comparing 100% strength to 50% dilution). In addition to the negative effect of high strength supernatants, increased

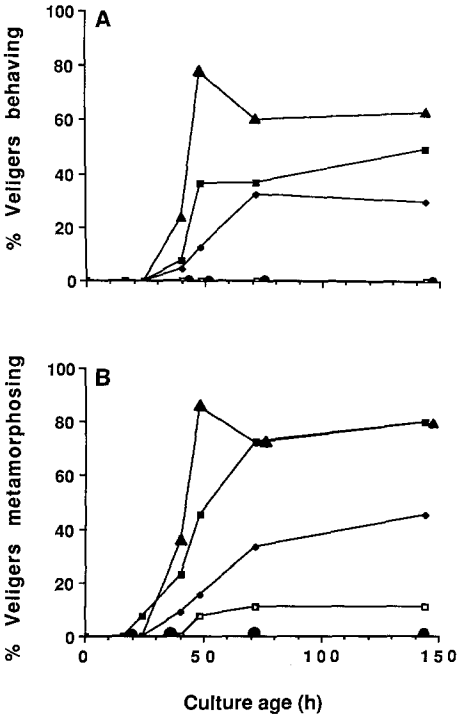

Fig. 1. *Crassostrea gigas.* Response of veligers to different concentrations of supernatants from different aged cultures of *Alteromonas colwelliana* grown in AG-medium. Concentration of bacterial supernatants diluted with seawater were as follows: (●) 0%; (□) 10% (◆) 25%; (■) 50%; (▲) 100%. (A) Settlement behavior; (B) metamorphosis

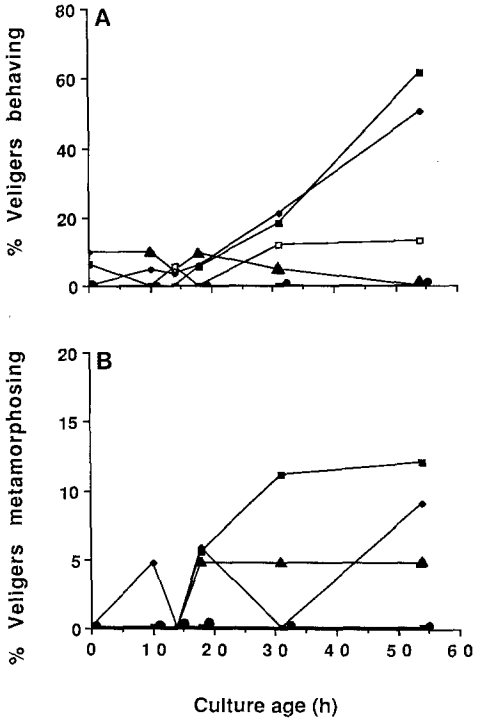

Fig. 2. *Crassostrea gigas.* Response of veligers to different concentrations of supernatants from different aged cultures of *Alteromonas colwelliana* grown in Marine Broth. Concentration of bacterial supernatants diluted with seawater were as follows: (●) 0%; (□) 10%; (◆) 25%; (■) 50%; (▲) 100%. (A) Settlement behavior; (B) metamorphosis

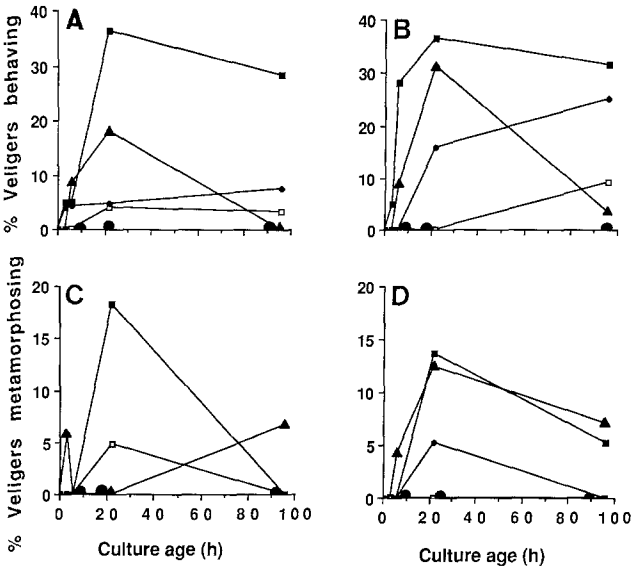

Fig. 3. *Crassostrea gigas.* Response of veligers to different concentrations of supernatants from different aged cultures of either pigmented [Strain HTX; (A and C)], or non-pigmented [Strain 596B; (B and D)], *Vibrio cholerae* grown in Marine Broth. Concentration of bacterial supernatants diluted with seawater were as follows: (●) 0%; (□) 10%; (◆) 25%; (■) 50%; (▲) 100%. (A and B) Settlement behavior; (C and D) metamorphosis

culture age could also be inhibitory. Supernatants at 100% strength and 50% dilution from the final sampling time of both pigmented and non-pigmented *V. cholerae* grown in MB induced less larval settlement behavior than supernatants from younger cultures (Figs. 3A, B and 4; $p<0.01$, paired t-test comparing final to preceding sample time), though the age of these cultures had no consistent effect on larval metamorphosis (Figs. 3C, D and 4; $p>0.5$, paired t-test comparing final to preceding sample times). There was no negative effect of culture age in supernatants from *A. colwelliana* grown in either MB or AG medium (Figs. 1 and 2; $p>0.5$, paired t-tests comparing final to any other sampling time). The decreased level of larval settlement behavior in response to stronger concentrations of older bacterial supernatants appears to be more severe in supernatants from the pigmented *A. colwelliana* and *V. cholerae* HTX compared to the non-pigmented *V. cholerae* 596-B. Based on the results above, all further experiments involving cultures of any age grown in MB were performed using 50% supernatant diluted with seawater.

Response of veligers of different ages from two different larval batches

Veligers of *Crassostrea gigas* become competent to exhibit settlement behavior in response to L-DOPA before they are competent to metamorphose in response to EPI or L-DOPA (Coon et al. 1990). To test whether larvae would show the same temporal separation of behavioral and morphogenetic competence in response to bacterial supernatants, veligers of two ages (Groups B and C3) were exposed to supernatants from cultures of the two

Table 1. *Crassostrea gigas.* Percentages of two different age groups of larvae exhibiting settlement behavior and subsequently metamorphosing in response to: (a) supernatants from cultures of *Alteromonas colwelliana* grown in AG medium; (b) L-3, 4-dihydroxyphenylalanine (L-DOPA); (c) epinephrine (EPI); (d) sterile AG medium; and (e) filtered seawater. Data are expressed as mean ± SD; ($n=6$). "Old" veligers (Group B at 32-d-old) were approximately 5-d older than "young" veligers (Group C 3 at 27-d-old). Comparisons of means between % Behaving and % Metamorphosing conducted using Student's t-test: $^{*}p<0.07$; $^{**}p<0.025$; $^{***}p<0.01$; $^{ns}p>0.1$. (–) no data

Inducer	Young veligers		Old veligers	
	Behaving	Metamorphosing	Behaving	Metamorphosing
A. colwell[a]	70.3 ± 11.5	44.5 ± 9.5***	66.9 ± 9.3	71.2 ± 15.4[ns]
L-DOPA				
1 m*M*	79.2 ± 4.3	64.7 ± 11.9**	82.1 ± 2.1	72.8 ± 14.1[ns]
100 μ*M*	80.2 ± 2.0	70.5 ± 10.9*	83.4 ± 3.2	74.9 ± 17.0[ns]
EPI 33 μ*M*	–	78.5 ± 8.0	–	100
AG medium	0	0 [ns]	0	0 [ns]
Seawater	0.6 ± 1.3	0 [ns]	1.8 ± 4.4	0 [ns]

[a] Data pooled from 48, 72 and 144-h old culture supernatants tested at 100% strength diluted in seawater

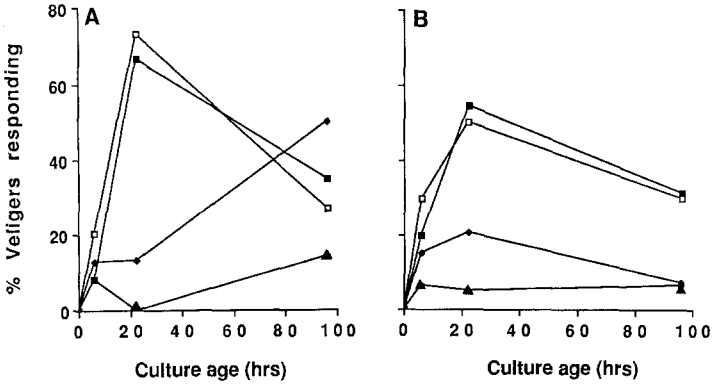

Fig. 4. *Crassostrea gigas.* Responses of two different age groups of veligers to supernatants from cultures of either pigmented [596 B; (A)], non-pigmented, or [HTX; (B)], strains of *Vibrio cholerae* grown in Marine Broth. "Old" veligers (■, ◆; Group B at 32-d-old) were approximately 5-d older than "young" veligers (□, ▲; Group C 3 at 27-d-old). Settlement behavior (■, □); metamorphosis (◆, ▲)

strains of *Vibrio cholerae* grown in MB (Fig. 4) and *Alteromanas colwelliana* grown in AG medium (Table 1). In response to supernatants from *V. cholerae,* older veligers exhibited higher levels of metamorphosis than the younger veligers (Fig. 4; $p<0.05$, paired t-test comparing all responses between young and old veligers), even though the percentage of oysters showing settlement behavior was similar for veligers of both ages (Fig. 4; $p>0.2$, paired t-tests comparing all responses between young and old veligers). Similarly, in response to supernatants from *A. colwelliana,* fewer young veligers metamorphosed than had exhibited settlement behavior (Table 1; $p<0.01$, t-test comparing responses within ages), while for old veligers the percentage metamorphosing was the same as the number exhibiting settlement behavior (Table 1; $p>0.1$, t-test comparing responses within ages). A similar age-dependent response was observed when the same two groups of larvae were exposed to L-DOPA (Table 1; t-test comparing responses within ages). This indicated that while competence to exhibit settlement behavior was similarly developed in both groups, competence to metamorphose in response to either bacterial supernatants or L-DOPA was more developed in older veligers.

Response of veligers to culture media and seawater

A low level of settlement behavior (mean ± 95% confidence interval = 7.4 ± 2.9%; $n=21$, pooled from all experiments) but no subsequent metamorphosis occurred in veligers exposed to autoclaved MB (data not shown). There was typically little settlement behavior and no metamorphosis observed in veligers exposed to either autoclaved AG medium or filtered seawater (Table 1).

Response of veligers to fractionated supernatants

When supernatants were fractionated according to molecular weight, fractions associated with only one UV-absorbing peak (Peak C) elicited behavior and metamorphosis of veligers (Fig. 5). The molecular weight of this peak was determined to be ≤ 300 daltons. This "active" peak was found in all supernatants of *Altermanas colwelliana* and both strains of *Vibrio cholerae* grown in MB (Fig. 6). The concentration of Peak C in raw supernatant was calculated to be ~ 5 mg ml^{-1}. In addition, Peak C from fractionated autoclaved MB induced low percentages of settlement behavior in veligers (Fig. 6). In contrast, while raw supernatants of *A. colwelliana* grown in AG medium exhibited strong inductive activity, no active peaks were found when these supernatants were chromatographed due to loss of inductive activity during lyophilization. No settlement behavior or metamorphosis was induced by fractionated AG medium with or without bacteria.

Discussion

The data presented here comprise the first experimental evidence that soluble chemicals produced in cultures of bacteria induce settlement and metamorphosis of oyster

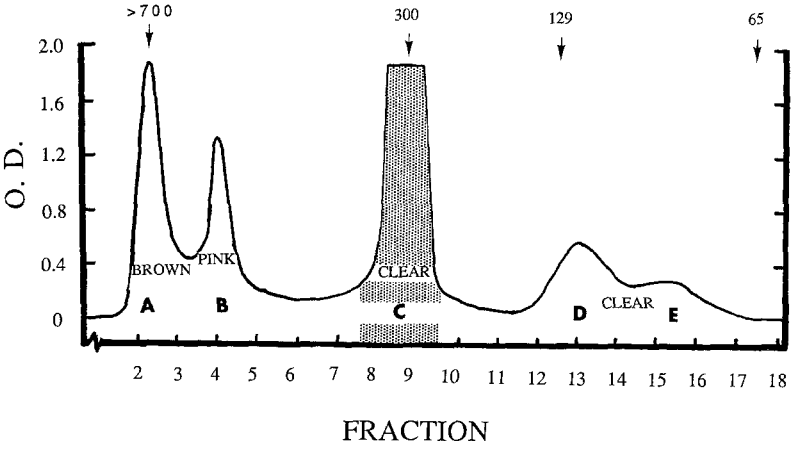

Fig. 5. *Alteromonas colwelliana.* Representative chromatogram of a fractionation of supernatant from cultures grown in Marine Broth (culture age = 27 h) on a Sephadex G-10 column (exclusion size molecular weight W > 700 daltons). Numbers denote additional MW standards. O.D. = optical density at 280 nm in arbitrary units. Inductive activity was found in hatched fractions. Peaks are lettered and their elutant color noted

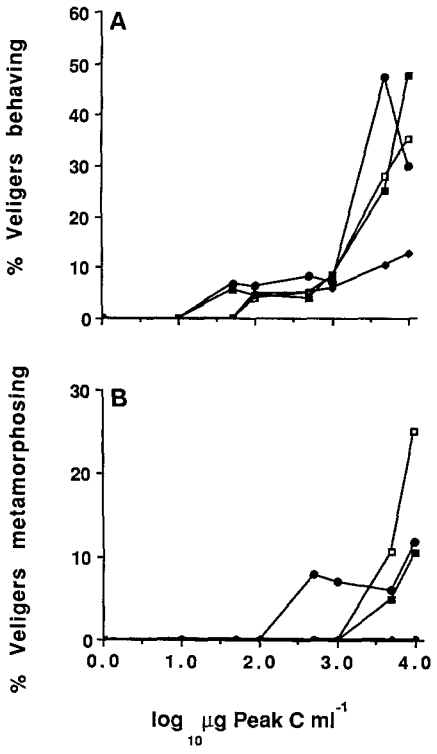

Fig. 6. *Crassostrea gigas.* Response of veligers to the active fraction (Peak C in Fig. 5) from supernatants from cultures of *Alteromonas colwelliana* (□), pigmented *Vibrio cholerae* HTX (■) and non-pigmented *V. cholerae* 596 B (●), all grown in Marine Broth, and from autoclaved Marine Broth control (♦). (A) Settlement behavior; (B) metamorphosis

larvae. Previous documentation of bacterial cues inducing settlement and/or metamorphosis of oyster larvae were provided by Crisp (1974) and Weiner et al. (1985), where the presence of bacterial films was correlated with increased set of larvae and the distinction between soluble and substrate-bound cues was unclear.

In a companion paper a model of the mechanism of settlement and metamorphosis of oyster larvae was proposed on the basis of veliger response to L-DOPA and EPI (Coon et al. 1990). The model detailed the onset of "metamorphic competence" in terms of the development of larval capabilities to exhibit settlement behavior in response to appropriate environmental cues and the capability to subsequently metamorphose. It was proposed that while soluble environmental cues could trigger settlement behavior, that cementation and subsequent metamorphosis involved achievement of another level of competence. For oysters this second requirement apparently involves perception of cues associated with particular substrates and an innate reduction of the response threshold associated with increasing age. Therefore, activation of the characteristic searching behavior in veligers exposed to bacterial supernatants does not necessarily also trigger metamorphosis. For this reason it is interesting to note that the response of different aged larvae to supernatants of cultures of *Altermonas colwelliana* in AG medium is almost identical to the response of the same larvae to L-DOPA (Coon et al. 1990, this study Table 1). The reason for the lack of correlation of larval response to supernatants from later stages of bacteria grown in MB with larval response to L-DOPA is not known but may be due to the build-up of noxious compounds in the MB, especially in cultures of pigmented bacteria, which are known to release quinones and other noxious intermediates in the chemical pathway leading to melanin (Nicolaus 1968). Alternatively, high concentrations of inducer may inactivate the larvae. Supernatants of *A. colwelliana* grown in AG medium may be less noxious than those grown in MB because final culture densities attained during stationary phase in AG medium are only 10% of those attained in MB (Fitt et al. 1989).

The active soluble chemical(s) in the bacterial supernatant has not yet been positively identified, but appears to be a small molecule (≤ 300 daltons), perhaps the same inducer found in autoclaved MB. Our observations of the disappearance of activity of the inducer from supernatants of cultures of *Altermonas colwelliana* grown in AG medium after lyophilization suggest that it may be labile under certain conditions.

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