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Molecules from cyanobacteria and red algae that induce larval settlement and metamorphosis in the mollusc *Haliotis rufescens*

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Abstract

Potent inducers of metamorphosis of planktonic larvae of the gastropod mollusc *Haliotis rufescens* have been found in the following phycobiliprotein-producing cyanobacteria: *Synechococcus* spp. (one marine and one freshwater strain), *Synechocystis* spp. (one hypersaline and one freshwater strain) and *Spirulina platensis* (a freshwater strain). No inducers were detected in the bacterium *Escherichia coli.* Inducers from one of the cyanobacteria (*S. platensis*) were partially purified and compared to inducers from the foliose red macroalga *Porphyra* sp. and the crustose coralline red alga *Lithothamnium californicum*. In all three species the inducers can be largely separated from the biliproteins, with which they appear to be associated, by high-resolution gel-filtration chromatography. The molecular weights of the relatively small inducing molecules resolved by these procedures from cyanobacteria and red algae are similar, falling in the range of 640 to 1 250 daltons. The amenability of the cyanobacteria to large-scale cultivation, and to physiological and genetic manipulation, make them useful for production of metamorphic inducers of marine invertebrate larval metamorphosis, and for further studies of the synthesis, structure and mechanism of action of such inducing molecules.

Introduction

Substratum-specific settlement, attachment and metamorphosis of the planktonic larvae of the Pacific red abalone *Haliotis rufescens* normally are induced by larval contact with molecules uniquely available to the larvae at the surfaces of crustose red algae (Morse and Morse, 1984a). The inducer in the intact algae and in cell-free algal extracts mimics the action of gamma-aminobutyric acid (GABA), and may be an algal metabolite homologous to GABA (Morse *et al.*, 1979, 1980a, b). Thus far, larvae of 12 species of *Haliotis* have been found to require contact with such algal inducers or GABA for induction of settlement and subsequent metamorphosis (Crofts, 1929; Shepherd, 1973; Morse *et al.*, 1979, 1980c; Saito, 1981; Morse, 1984a; Morse, Morse and Zhang, unpublished data). Larvae of a number of other molluscan species, including *Tonicella lineata* (Barnes and Gonor, 1973), *Mopalia muscosa* (Morse *et al.*, 1979), *Katharina tunicata* (Rumrill and Cameron, 1983), *Acmaea testudinalis* (Steneck, 1982), *Trochus niloticus* (Heslinga, 1981) and *Acmaea undosa* (Markell and Morse, unpublished data) also are induced to settle and metamorphose by related algae, algal extracts, or GABA.

In their natural environment, abalone larvae encounter the required inducing molecules at the surfaces of crustose red algae genera such as *Lithothamnium, Lithophyllum* and *Hildenbrandia* (Morse *et al.*, 1980c). These inducers apparently are transported to the surfaces of the crustose algae from the underlying algal cells (Morse and Morse, 1984a). Molecules with similar inducing activities were found in extracts of foliose red algae, including species of *Laurencia, Gigartina,* and *Porphyra,* although inducers were not found available to the larvae at the surfaces of any of these algae, nor were abalone larvae induced to settle on the intact fronds of these foliose species (Morse and Morse, 1984a, b). Abalone larvae are not induced to metamorphose by contact with foliose green or brown macroalgae or their cell-free extracts. These findings, together with the observation that in cell-free extracts the inducer from red algae appears to be associated or complexed with phycoerythrin or other phycobiliprotein molecules (Morse *et al.*, 1979; Morse and Morse, 1984a, b) suggest that the inducing molecules found in crustose and foliose red algae are associated with the phycobiliproteins, their precursors, or degradation products. Partial purification by ion-exchange chromatography resolved the principal inducing molecules from the phycobiliproteins of these algae, although some residual inducing activity still remained with the biliprotein component (Morse and Morse, 1984a, b).

The association of these inducers with the phycobiliproteins suggested that, because of the close biochemical and phylogenetic relationship between the red macroalgae and the biliprotein-producing cyanobacteria (blue-green algae) (Bonen and Doolittle, 1975, 1976; Stanier and Cohen-Bazire, 1977), similar inducers of molluscan larval metamorphosis might be present in the cyanobacteria. We report here the finding of inducers of abalone settlement and metamorphosis from 5 different strains of marine, halophilic and freshwater cyanobacteria of the genera *Synechococcus, Synechocystis* and *Spirulina.* Inducer was not found in a non-photosynthetic gram-negative bacterium, *Escherichia coli.* The inducing molecules from *Spirulina platensis* have been partially purified, and found to be similar to those obtained by similar procedures from *Porphyra* sp. and *Lithothamnium californicum.*

Cyanobacteria are easily grown on a large scale and easily manipulated physiologically and genetically (Shestakov and Khyen, 1970; Kuhlemeier *et al.*, 1981; Sherman and van de Putte, 1982; Williams and Szalay, 1983). Therefore, cyanobacteria may prove to be especially useful sources of inducing molecules for the control of marine invertebrate metamorphosis, and for further studies of the synthesis, nature and mechanism of action of such morphogenetic inducers.

Materials and methods

Cyanobacteria and algae

The marine cyanobacterium *Synechococcus* Strain ATCC # 27265 (formerly *Coccochloris elabens*), the halophilic cyanobacterium *Synechocystis* Strain ATCC # 27266 (formerly *Eucapsis* sp.), and the two freshwater cyanobacteria *Synechococcus* Strain ATCC # 27144 (formerly *Anacystis nidulans*) and *Synechocystis* Strain ATCC # 27178 (formerly *Aphanocapsa* sp.) were obtained from the American Type Culture Collection, Rockville, Maryland, USA, and cultured phototrophically by the methods of Rippka *et al.* (1979). *Escherichia coli* (Strain K12-W3110) was cultured in V-B minimal medium as described by Miller (1972). Cells were harvested and washed twice with buffer (described below) by centrifugation for 10 min at 15 000 × *g* at 2 °C. *Spirulina platensis* (Mexican Lake Texococo strain) was obtained dried from Pacific International Labs Inc., Orange, California. A dried species of *Porphyra* from Japan (Nori) was purchased commercially; monocultures of *Lithothamnium californicum* were maintained and identified as previously described (Morse and Morse, 1984a).

Cell-free extracts

Cell-free extracts of the cyanobacteria and *Escherichia coli* were prepared by suspension of the cells in 50 m*M* tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), pH 8, containing 0.17 *M* sodium chloride (NaCl) (3 ml of buffer per gram of wet cells, or 40 ml per gram of dry cells), followed by disruption for 1 min at 0 °C with either a Branson sonifier (Ultrasonics, Inc., Plainview, New York) or a Janke and Kunkel Ultra-Turrax cell disruptor (Model SDT, Tekmar Co., Ohio). The disrupted cells were centrifuged at 24 000 × *g* for 10 min at 2 °C, and the resulting supernatant (crude extract) kept on ice until assayed or further purified. Extracts of *Porphyra* sp. and *Lithothamnium californicum* were prepared as described previously (Morse and Morse, 1984a), except that 50 m*M* Tris-HCl buffer at pH 8 containing 0.17 *M* NaCl was used.

Gel-filtration chromatography of inducers

Crude extracts (12 ml containing 2.4 mg ml^{-1} protein) of *Spirulina platensis, Porphyra* sp. and *Lithothamnium californicum* were fractionated separately by chromatography on a column (2.6 × 55 cm; 300 ml bed volume) of Ultrogel AcA 202 (LKB Instruments, Inc., Gaithersburg, Maryland), a gel-filtration resin designed for high-resolution of low molecular weight molecules. The column was equilibrated and eluted with 50 m*M* Tris-HCl buffer at pH 8, containing 0.17 *M* NaCl. Fractions of 3 ml were collected and 1 ml of each fraction then assayed for biological activity to identify the inducing molecules. A closed column system and pump were employed to ensure reproducibility of fractionation. A crude extract of *L. californicum* also was chromatographed on Ultrogel AcA 34 resin, all other conditions being identical to those employed for fractionation on AcA 202.

Partially purified inducers from *Spirulina platensis* and *Lithothamnium californicum* (≦ 1 500 daltons) obtained by gel-filtration on Ultrogel AcA 202 and AcA 34, respectively, were further resolved on a column (2.6 × 38 cm; 200 ml bed volume) of Sephadex-G10 (Pharmacia), equilibrated and eluted with Tris-HCl buffer (10 m*M*, pH 8). The column was pre-calibrated with blue dextran and two low molecular weight markers, Vitamin B_{12} and potassium dichromate (see next section), applied in the same buffer volume as the samples (20 ml). Fractions of 2 ml were eluted, and 1 ml of each was assayed for biological activity.

Determination of molecular weights of inducers

The molecular weights of the principal inducing molecules from *Spirulina platensis, Porphyra* sp., and *Lithothamnium californicum* were determined by gel-filtration on Ultrogel AcA 202 (same conditions as described above). Individual column pre-calibrations were performed for each of the three extracts with known molecular weight standards (bovine serum albumin, from Pharmacia, mol. wt = 67 000 daltons, used to determine the excluded volume; cytochrome C, from Boehringer Mannheim, mol. wt = 12 500 daltons; Vitamin B_{12}, from Sigma Chemical Co., mol. wt = 1 350 daltons; potassium dichromate, from Sigma

Chemical Co., mol. wt = 294 daltons). From the elution volumes observed, the molecular weights of the biologically active compounds were calculated by the method of Andrews (1965).

Membrane filtration of inducers

Low molecular weight inducing molecules from *Spirulina platensis* and *Lithothamnium californicum* were obtained by filtration on Ultrogel AcA 202 and 34, respectively, further fractionated on Sephadex G10, and then separated by filtration through a Diaflo Ultrafilter, Type YCO5 membrane, using an Amicon filtration apparatus under 40 psi of nitrogen, with constant stirring at 0 °C. Equivalent volumes of pre-filtered, retained and filtrate solutions were then assayed for biological activity and protein.

Protein and spectral characterization

Algal extracts and all fractions obtained by column chromatography were assayed for protein by the method of Bradford (1976); chromophore content was measured with a Gilford UV-visible spectrophotometer to obtain absorbance values for phycoerythrin at $\lambda_{max} = 562$ nm. Absorption spectra for the phycobiliproteins were obtained as previously described (Morse and Morse, 1984 a).

Induction of molluscan settlement and metamorphosis

The assay organisms were 8 to 9 d-old larvae of *Haliotis rufescens* produced and reared in the laboratory (Morse *et al.*, 1977, 1979). These lecithotrophic larvae required no feeding during the course of the experiments. All assays were conducted in clean glass vials at 15 °C ± 1 C°, in 10 ml of filtered, ultraviolet-sterilized seawater containing 150 mg l^{-1} each of penicillin G and dihydrostreptomycin, with approximately 200 competent planktonic abalone larvae and additions as noted. The percentage of larvae induced to attach to the glass substratum ("settlement") and to undergo metamorphosis was determined by microscopic examination. Parallel duplicate controls included in each experiment indicated that in the presence of GABA at 10^{-6} *M*, settlement was always greater than 85% by 24 h, while in the absence of any additions, settlement was ≦ 1% (± 1%) for the duration of the experiments.

Results

Metamorphosis inducers in cyanobacteria

Inducing activity was detected in crude extracts of all 5 strains of cyanobacteria tested (Table 1). No inducing activity was detected in extracts of the unrelated bacterium, *Escherichia coli*. In contrast to intact *Lithothamnium*

Table 1. *Haliotis rufescens.* Attachment of abalone larvae after 20 h, in response to additions shown. Two samples of 200 ± 50 larvae each were counted for each addition. Results with *Lithothamnium californicum* extract are for 20 µg protein ml^{-1}; results for all other extracts are for concentrations of 100 µg ml^{-1}. Intact bacteria were sufficient to uniformly cover bottoms of assay vials; results with intact *L. californicum* are from Morse and Morse (1984 a)

Addition	Attachment (% ± SD)
None	0 ± 0
Cell-free extracts:	
Lithothamnium californicum	97 ± 2
Synechococcus ATCC #27265	100 ± 0
Synechococcus ATCC #27144	83 ± 11
Synechocystis ATCC #27266	24 ± 5
Synechocystis ATCC #27178	17 ± 7
Spirulina platensis	96 ± 2
Escherichia coli	0 ± 0
Intact:	
Synechococcus ATCC #27265	0 ± 0
Synechococcus ATCC #27144	0 ± 0
(*Lithothamnium californicum*)	100 ± 0

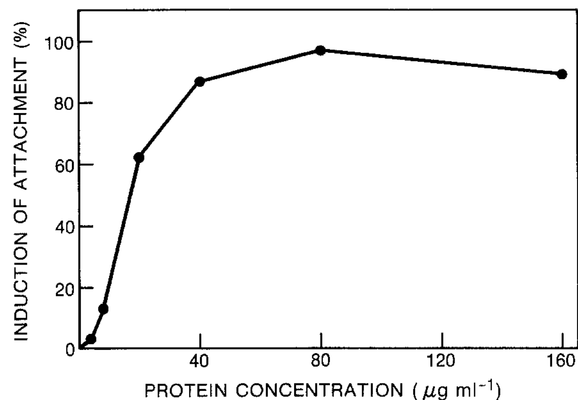

Fig. 1. *Haliotis rufescens.* Concentration-dependent induction of larval abalone attachment by a crude extract of the cyanobacterium *Spirulina platensis.* Extract was assayed at the final concentrations indicated; each point is an average of duplicate samples, assayed at 16 h; standard deviation from mean was ≦ 3% in each sample set. Duplicate controls with no additions and with 10^{-6} *M* GABA (gamma-aminobutyric acid) yielded 0% (± 0) and 74% (± 7), respectively

californicum, however, intact cyanobacteria induced no attachment of *Haliotis rufescens* larvae (Table 1), indicating that the cyanobacteria neither release soluble inducers, nor bear inducers at their surfaces. Similar results were obtained previously for foliose red algae (Morse and Morse, 1984 a). Because *Spirulina platensis* is commercially available in large quantities, it was chosen for further biochemical studies.

Fig. 1 illustrates the dependence of inducing activity upon the concentration of *Spirulina platensis* extract added. This material induced metamorphosis (abscission of the velar lobes, and commencement of growth of the

juvenile shell) in virtually all of the larvae induced to settle. Such metamorphosis was observed within 40 to 48 h after addition of the inducer, a time-course similar to that for metamorphosis induced by the active component from red algae. The concentration of extracts of *S. platensis* and the marine *Synechococcus* strain (measured in terms of protein) required for induction of ca. 100% of the larvae tested, in a number of independent tests, was consistently 2 to 5-fold higher than that of an extract of *Lithothamnium californicum* required to cause the same level of induction (cf. legend to Table 1).

Inducing molecules from cyanobacteria and red algae partially purified by gel-filtration

Previously we reported that, under mild extraction conditions (10 m*M* Tris-HCl, pH 7 or pH 8), the inducing activity from *Lithothamnium* spp., *Porphyra perforata, Laurencia pacifica* and *Porphyra* sp. (Nori) is associated with the macromolecular fraction of these algal extracts after gel-filtration on Sephadex G-25 (Morse and Morse, 1984a). We further demonstrated, however, that the inducing activity could be separated from the major protein component (phycobiliprotein) by further purification on DEAE Trisacryl-M (Morse and Morse, 1984a, b). We demonstrate here that, by increasing the salt concentration of the buffer (50 m*M* Tris-HCl, pH 8 + 0.17 *M* NaCl) and selection of a more specific gel-filtration medium (see "Materials and methods"), we can, in one step, obtain the majority of the inducing species as a relatively low molecular weight component resolved from the biliproteins from crude extracts of *Spirulina platensis, Porphyra* sp. (Nori) and *Lithothamnium californicum* (Fig. 2). This ease of separation in the presence of a higher concentration of salt suggests that the inducing molecules may be non-covalently bound to cyanobacterial or algal proteins. Although separation of the majority of the inducing molecules with comparably high activity from *S. platensis* and *Porphyra* sp. is readily and reproducibly achieved using Ultrogel AcA 202 (Fig. 2A, B), it proved impossible to obtain a high yield of relatively low molecular weight inducing molecules from *L. californicum* with this gel-filtration resin (Fig. 2C). It was necessary to utilize a resin with a higher molecular weight fractionation range (Ultrogel AcA 34) for more efficient separation of low molecular weight inducing molecules from the biliproteins of *L. californicum* (Fig. 2D). This result suggests that the inducing molecules may be complexed differently or more strongly to macromolecules of the crustose algae than to those of the cyanobacterial and foliose algal species.

The resulting low molecular weight inducing molecules from all three species are colorless (no absorption λ_{max} in the visible range; $A_{562} = 0$ at 10× concentration of the eluted fractions), contain no protein and are well separated from the phycobiliproteins whose elution positions are shown by the horizontal arrows in Fig. 2. The biological activity of these molecules, as measured by induction of settlement and metamorphosis of competent abalone

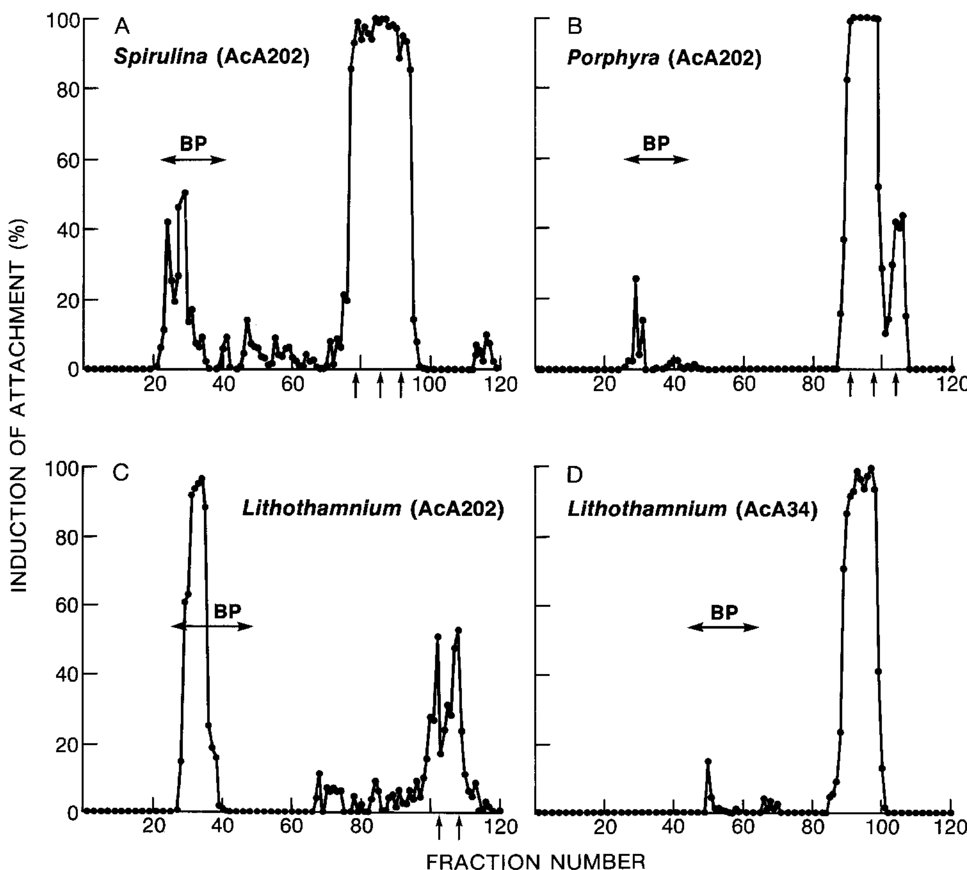

Fig. 2. *Spirulina platensis, Porphyra* sp. and *Lithothamnium californicum.* Low molecular weight inducers from the 3 sources, (A)–(C), show similar distributions on gel-filtration on Ultrogel AcA 202. Inducers from *L. californicum* were also chromatographed on Ultrogel AcA 34; their distribution is shown in (D). Crude extracts were prepared and chromatographed as described in "Materials and methods"; portions (1 ml) of each chromatographic fraction were assayed for inducing activity after 40 h (●); duplicate controls with no additions yielded 0% (±0) attachment in (A)–(D); those with 10^{-6} *M* GABA yielded 98% (±0.5), 93.5% (±0.5), 95% (±2.0) and 92% (±1) for (A), (B), (C) and (D), respectively. Vertical arrows on *x*-axis of (A)–(C) indicate peak fractions of inducers for which molecular weights were determined. Elution position of phycobiliproteins (BP) of each extract is shown

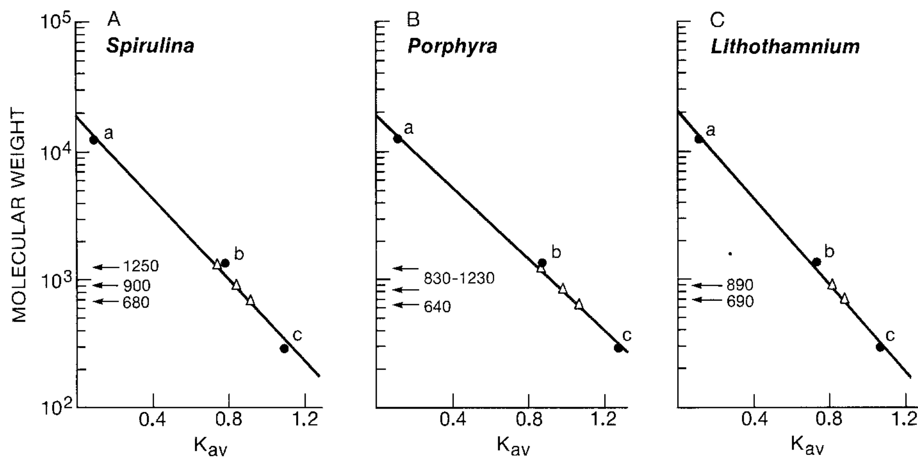

Fig. 3. *Spirulina platensis, Porphyra* sp. and *Lithothamnium californicum*. Estimated molecular weights (in daltons) of principal inducers (△) [peaks indicated by vertical arrows on *x*-axis of Fig. 2 (A)–(C)] from each of the three species, determined by gel-filtration on Ultrogel AcA 202. Each analysis was calibrated independently with the relative elution volumes (K_{av}) of (a) cytochrome C, (b) Vitamin B_{12}, (c) potassium dichromate, and the log of their molecular weights by linear regression

larvae, is comparable for the three species tested (Fig. 2A, B, D). In the extracts of each of these three species, there are at least two principal and distinct inducing components in the low molecular weight range. The double peaks of low activity seen associated with the high molecular weight fractions of *Spirulina platensis* and *Porphyra* sp. were determined, by spectrophotometric scanning of samples, to be associated with the phycoerythrin and phycocyanin components of these extracts; the single high molecular weight peak of activity of *Lithothamnium californicum* is associated with the phycoerythrin of this alga (Fig. 2, and results not shown).

Inducers with similar molecular weights from cyanobacteria and red algae

Results of gel-filtration molecular weight analysis of the most potent low molecular weight inducing molecules from *Spirulina platensis, Porphyra* sp. and *Lithothamnium californicum* reveal that the inducers from each of the three species have molecular weights which fall within the same narrow range (Fig. 3). Values of 1 250, 900 and 680 daltons for *S. platensis* represent the apparent molecular weights of Fractions 79, 86 and 92, respectively, as shown in Fig. 2A. From *Porphyra* sp., the major inducing activity exhibits apparent molecular weights ranging from 1 230 to 830 daltons (with separate species not resolved within this range, corresponding to Fractions 91–98 in Fig. 2B); an active component of ca. 640 daltons corresponds to Fraction 104. From *L. californicum*, apparent molecular weights of 890 and 690 daltons were determined (Fractions 102 and 108 in Fig. 2C). The distributions of apparent molecular weights of the inducing species from *S. platensis* and *L. californicum* described above were confirmed by gel-filtration on Sephadex G-10, and by ultrafiltration through low molecular weight-retention membranes (see "Materials and methods").

Discussion and conclusions

The results presented here confirm the prediction that marine, halophilic and freshwater cyanobacteria contain molecules that induce the settlement and metamorphosis of abalone larvae. With these observations, *Haliotis rufescens* joins the number of marine invertebrates for which larval settlement and metamorphosis have been shown to be induced by metabolites of specifically identified bacterial cells (e.g. Müller, 1969; Kirchman *et al.*, 1982; Weiner and Colwell, 1982). In *H. rufescens*, however, these observations probably are of little, if any, direct ecological significance to primary settlement in the natural environment, because the intact cells of the cyanobacteria are non-inductive. Furthermore, we have demonstrated that inducers partially purified by gel-filtration from *Spirulina platensis* have molecular weights and biological activity similar to those of inducers from the foliose red alga *Porphyra* sp. and the natural substratum *Lithothamnium californicum*. The inducing molecules from all 3 species, after separation from the phycobiliproteins, are colorless and contain no protein.

The results of this study extend our previous findings, confirming that the inducers of abalone settlement and metamorphosis are in some way associated with the phycobiliproteins. These inducers are found in extracts of all five of the cyanobacteria tested, as well as in extracts of all of the red algae tested, but are not in *Escherichia coli* or in any of the green or brown algae studied (Morse and Morse, 1984a). In addition, the lack of morphogenetic activity of the intact cyanobacteria confirms the conclusion that it is the unique availability of the inducing molecules at the surfaces of the crustose red algae (demonstrated directly in our previous study; cf. present Table 1) that is responsible for the substrate-specificity of larval settlement and metamorphosis on these algae (Morse and Morse, 1984a).

We anticipate that the tractability of the cyanobacteria for physiological and genetic manipulations, and the availability of large amounts of inducing molecules from cyanobacterial sources, will facilitate further studies on the synthesis, structure and mechanism of action of the chemical inducers of molluscan larval settlement and metamorphosis. In addition, the present findings are directly applicable to three related areas of marine research and development: (1) the synchronous control of settlement and metamorphosis of larval cultures, for improved culti-

vation of experimentally or commercially important marine invertebrate species (Morse, 1984a); (2) the analysis of mechanisms controlling marine invertebrate recruitment and marine fouling, to which methods of bacterial genetics and gene cloning can now be applied to study the production, structure and function of cyanobacterial inducers of settlement and metamorphosis (Morse, 1984b); and (3) the production and analysis of a new class of potent GABA-mimetic substances of potential biomedical importance (Morse, Roberts and Morse, unpublished data).

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