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Evidence for the Occurrence of Specific Iron (III)-Binding Compounds in Near-Shore Marine Ecosystems

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Aqueous extracts of samples from representative types of near-shore marine ecosystems were examined for growth-promoting activity for the siderochrome auxotroph, *Arthrobacter* JG-9. Blue-green algal mat and sea grass samples gave strongly positive responses. Relative to Desferal (Ciba Pharmaceutical Co., Summit, N.J.), concentrations up to 110 ng/g (wet weight) of sample were found. In contrast, extracts of anaerobic muds or macroalgae gave no detectable activity. The hydroxamate type of iron chelator may be important in providing a form of iron in sea water readily utilized by the microalgae and marine higher plants.

There has accumulated over the past 20 years an impressive body of information on microbial assimilation of iron and there now exist many examples of specific microbial iron (III)-chelating compounds or siderochromes. The biological aspects of this area have been recently reviewed by Lankford (6), whereas aspects of the chemistry of specific iron-binding compounds have been reviewed by Neilands (7). An obvious extrapolation of this body of information on iron uptake and metabolism by microorganisms can be made to marine ecosystems and to the problem of iron uptake in phytoplankton and marine plants. Spencer et al., largely on the basis of infrared spectra, have suggested that ferric-specific organic chelators are produced by marine phytoplankton cultures (8). Lange (5) has noted chelator-like activity in growth experiments using mainly cultures of blue-green algae which contain bacteria. He infers that these chelators may be related to the problem of iron uptake in the blue-green algae. The blue-green algae as a group have a strong preference for alkaline growth conditions, and it may be mandatory that they produce specific iron chelators. Hutner (4), in a provocative review on inorganic nutrition, has suggested that iron chelators may play a key role in phytoplankton growth and succession. In addition, a computer simulation of a nine-metal, nine-ligand system, chosen to mimic natural waters, illustrates that soluble iron (III) exists almost exclusively as a norcardamine (ferrioxamine) chelate (9).

We have used the well-described siderochrome auxotroph, *Arthrobacter* JG-9 (1, 2, 3), to examine extracts from near-shore marine ecosystems for growth factor activity for this organism. It is an assumption of this work that growth-promoting activity in an extract indicates the presence of certain secondary hydroxamic acids (specific iron chelators) in the sample. JG-9 will also show a growth response to hemin, aspergillic acid, kojic acid, 2,3-dihydroxy-*N*-benzoyl-L-serine (6); however, we discount these compounds as a potential source of activity since the concentrations required to support growth for JG-9 seem unrealistic for natural systems.

MATERIALS AND METHODS

Assay organism and growth conditions. *Arthrobacter* JG-9 was obtained from C. E. Lankford, Microbiology Dept., University of Texas, Austin. The medium was composed of three solutions autoclaved separately and mixed after cooling to 50 C. Solution A contained per 450 ml of distilled water: K_2HPO_4, 2 g; $(NH_4)_2PO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; yeast extract, 1.0 g; Casamino Acids, 1.0 g. Solution B contained 10 g of sucrose in 100 ml of distilled water and solution C, 8 g of Bio-Rad agarose powder (no. 1620100) in 450 ml of distilled water. The pH was adjusted to 7.6 before autoclaving. For routine growth and stock slants of JG-9 the medium was supplemented with 100 μg of Desferal, methane sulfonate of iron-free ferrioxamine B (Ciba Pharmaceutical Co., Summit, N.J.) per liter. The organism was depleted of siderochrome by inoculating a loopful from a slant into 10 ml of the above medium minus agarose and minus Desferal and grown on a shaker for 48 h. The cultures was then diluted to approximately 10^7 cells per ml and a 1/100 dilution of this suspension was used as an inoculum for petri dishes containing 20 ml of agar medium. The Desferal standards were made by dilution of a known weight. Both standards and the unknown extracts were absorbed (~0.1 ml) into S&S (no. 740-E) 13 mm, distilled water-washed, filter paper disks. The pads

were applied directly to seeded JG-9 plates and the plates were incubated at 25 C. After 48 h growth measured as diameter of zone was recorded relative to the Desferal standards. Zone diameter was proportional to log of Desferal concentration from 10 to 1,000 ng/ml.

Extraction procedures. The samples were collected and processed immediately or frozen until used. The mud and blue-green algal mat samples were homogenized by sonication, Branson model S 125 at a setting of 6, tuned for maximum output. Distilled water was added as needed to liquefy the sample and the resulting slurry was stirred continuously by a magnetic stirrer during the sonication period, 30 to 60 min. The macroalgae or grass samples were minced slightly and placed in distilled water. Both the mud-mat extracts and the algal-grass type samples were autoclaved at 121 C for 30 min. After cooling, the liquid was separated by filtration through acid-washed Celite. The filtrate was concentrated under vacuum below 60 C and kept frozen until assayed (sample extraction method 1 in Table 1). These concentrated aqueous extracts were reautoclaved before assay. With some of the aqueous extracts an attempt was made to concentrate the active materials on charcoal (Table 1, sample extraction method 2). Water-washed charcoal was added to the aqueous extract, the pH was adjusted to approximately 4 with acetic acid, and the whole was mixed at intervals. After standing overnight, the charcoal was recovered by filtration on a Corning, medium porosity, sintered glass filter, and eluted with *n*-propanol, 2.85% NH_4OH, 2:1 (vol/vol). The propanol-ammonia eluate was evaporated to dryness and the residue was dissolved in distilled water to the final volumes shown in Table 1.

RESULTS AND DISCUSSION

We take as prima facie evidence that a JG-9 growth response to an extract indicates the presence of secondary hydroxamic acids, that is,

TABLE 1. *Arthrobacter JG-9 growth response to extracts of near-shore marine samples*

Sample no. and type	Sample characterisitics	Sample size (g [wet wt])	Sample extraction method	Initial and final (assay) sample vol (ml)	Nanograms of JG-9 responsive material/g of sample
1. Blue-green algal mat	Dry mat on land	90	1	100–20	23
		70	2	300–4	2
2. Blue-green algal mat	Moist, recurring mat area, on land	270	2	300–4	15.1
3. Blue-green algal mat	Floating mat, small pond on land	90	1	100–20	39
		270	2	300–4	0
4. Blue-green algal mat	Redfish Bay, shallow water	60	1	100–25	18
5. Decayed blue-green algal material	Greenish-orange floating clumps from concrete holding tank	440	2	440–4	3.2
6. Blue-green algal mat	Fresh mat, flooded land area	140	1	140–30	10
7. Mud plus filamentous green alga, Diatoms	Overflow from sea water ponds	60	1	100–15	20
8. Decaying *Thalassia and Halodule*	Redfish Bay, at edge of water	85	1	150–15	110
9. *Halophila* and *Halodule*	Redfish Bay, healthy plants	20	1	50–1	5
10. *Thalassia*	Floating in water, decaying, highly epiphytized	165	2	150–4	4
11. *Sargassum*	Fresh, on Gulf beach	75	1	100–20	0
		190	2	250–4	0
12. *Enteromorpha*	Fresh, on rocks at jetty	100	1	100–20	0
		300	2	300–4	0
13. *Porphyra*	Fresh, on rocks at jetty	66	1	100–20	0
		120	2	180–4	0
14. *Ulva*	Fresh, on rocks at jetty	100	1	100–20	0
		250	2	250–4	0
15. Anaerobic mud	Aransas Pass boat basin, heavy H_2S odor	130	1	100–20	0
		400	2	300–4	0
16. Anaerobic mud	Very black layer beneath algal mat, heavy H_2S odor	100	1	100–20	0
		250	2	250–4	0
17. Aerobic sediment	Largely sand, brownish gray	600	2	350–4	0
18. High dune sand	Clean, no plants	120	1	100–20	0
		420	2	350–4	0

specific iron (III)-binding compounds (1, 2, 3, 6). With this in mind the data in Table 1 show considerable activity with autoclaved aqueous extracts from the blue-green algal and sea grass samples, in fact, better than the charcoal processed extracts. The amounts found, nanograms per gram relative to Desferal, are not insignificant. Such iron-binding compounds are known to be active as growth factors at very low concentrations, 10^{-9} M (3). In the case of the macroalgal samples and the mud samples, either iron uptake in these systems does not depend upon the types of compounds active for JG-9 or the concentrations present were below the limits of detection of the assay.

Paper chromatography using Whatman 3MM paper, with *n*-propanol, 1% NH_4OH 2:1 (vol/vol), clearly showed that the compounds stimulating growth of JG-9 were not Desferal. In the extracts examined so far, samples no. 1, 2, 5, and 10 showed discrete zones of JG-9 activity at R_f values different from Desferal.

It is interesting that two types of ecosystems common to shallow, temperate, near-shore marine environments; blue-green algal mats and sea grass beds, gave strongly positive results in the JG-9 assay. In both these types of ecosystems we expect high bacterial activity, and this may be the source of the JG-9 activity found. Coryneform bacteria in particular may be the source (10). On the other hand we have isolated, purified, and partially characterized a compound from a pure culture of the blue-green alga, *Agmenellum quadruplicatum*, strain PR-6, which is JG-9 active and has chemical characteristics indicating that it is a secondary hydroxamate (unpublished observations). For the time being, we make no specification as to the sources of the activity found. The main point is that sufficient JG-9 active material is present in the blue-green algal mat and sea grass ecosystems to lead us to suggest that in some types of microalgae and marine plants, iron uptake may depend upon specific iron chelators of the hydroxamate type. As a corollary to this, such compounds could also be influencing species diversity through their known antibiotic activity (6).

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