STUDIES ON BACTERIAL UTILIZATION OF URONIC ACIDS

IV. ALGINOLYTIC AND MANNURONIC ACID OXIDIZING ISOLATES[1]

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Several species of bacteria are known to utilize galacturonic and glucuronic acids (Thofern and Hollmann, 1956; Payne and Carlson, 1957; and McRorie, Williams, and Payne, 1959), but there have been no reports of utilization of mannuronic acid. DeLey (1953) and Starr, DeLey, and Kilgore (1957) observed that *Aerobacter cloacae* and *Erwinia carotovora* did not metabolize this acid. We have found (Payne and McRorie, 1958) that uronic isomerase from *Serratia marcescens* produced barely detectable quantities of keturonic acid from mannuronic acid, but whole cells did not oxidize this alduronic acid. It seems likely, therefore, that mannuronate is metabolized differently from the other uronic acids.

Bacteria which hydrolyze alginic acid are known (Waksman, Carey, and Allen, 1934). Since mannuronic acid is the major hydrolytic product of this polymer, it seemed probable that these bacteria will utilize this substrate. That has proved to be true with two isolates obtained in our laboratory.

This paper describes the isolation and some properties of these two organisms, *Alginomonas alginica* from brown algae obtained at Sapelo Island, Georgia, and a less actively alginolytic pseudomonad from soil by enrichment culture. These bacteria oxidize mannuronate with constitutive enzymes, whereas galacturonic and glucuronic acids are not metabolized.

MATERIALS AND METHODS

Isolation and characterization of bacteria. A. alginica was isolated from a mass of brown algae which was ground in a mortar and streaked over a gel of 15 per cent sodium alginate in sea water. This crude culture was incubated at 25 to 30 C until fluid spots appeared in the gel. Several loopfuls of this viscous fluid were transferred to sea water nutrient agar for colonial isolation. Inocula from selected colonies were then transferred to a medium composed of 10 per cent sodium alginate, 0.01 per cent yeast extract, and 0.05 per cent $(NH_4)_3PO_4$ with sea water as diluent. The medium was sterilized by fractional steaming before use.

Colonies of the alginolytic bacteria associated with the algae sank into this medium. Pure cultures from these sunken colonies were identified as *A. alginica* by the usual methods of determinative bacteriology, except that culture media were prepared in sea water. It was found, however, that sea water is only stimulatory and not required for growth by *A. alginica*.

The unidentified pseudomonad (or alginomonad) was isolated from sunken colonies on a mineral salts-sodium alginate-yeast extract fresh water medium inoculated with soil enriched for 1 week with alginic acid after repeated attempts at direct isolation failed. It was not identifiable with any species in the genera *Pseudomonas* and *Alginomonas* described in the seventh edition of *Bergey's Manual of Determinative Bacteriology* (Breed, Murray, and Smith, 1957). Morphological and physiological characteristics of the isolate were determined by the usual methods.

Electron micrographs of both isolates were prepared with cells taken from agar slant cultures and shadowed with chromium.

Preparation of suspensions of resting cells. Inocula of *A. alginica* from 24-hr broth cultures were streaked over the surface of a minimal salts agar containing 0.5 per cent alginate and 0.01 per cent yeast extract or nutrient agar in large petri dishes. The cultures were incubated for 72 hr at 30 C. The cells were then scraped from the surface, washed twice in the suspending medium and suspended in 0.04 M mono- and dibasic sodium phosphate or disodium-monopotassium phosphate buffer at pH 7.0. The

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suspensions were adjusted to concentrations giving 5 per cent light transmittance at 420 mμ.

The use of agar grown cells was necessary because those grown in liquid culture could not be separated from residual alginate or clumped and did not suspend evenly.

Cells of the unidentified species were cultured for 24 hr at 30 C on a rotary shaker in nutrient broth or alginate agar-broth biphase (Tyrrell, MacDonald, and Gerhardt, 1958). The bacteria were centrifuged, washed twice and suspended in 0.04 M sodium or potassium phosphate at pH 7.0 as previously described.

Preparation of substrates. Alginic acid (Kelco) was dialyzed against distilled water in the cold, washed and collected on hard filter paper. The paste, free of reducing sugar, was air dried. This polymer was made soluble by the addition of NaOH to a 1 per cent suspension to give a pH of 8.0.

D-Mannuronolactone was prepared from alginic acid by the method of Soehr (1947). The lactone was converted to the sodium salt in solution with NaOH just before use. This preparation did not contain guluronolactone (Haug, 1959).

Assays of alginolytic and oxidative activity. Alginolysis was determined by preparing a series of reaction mixtures which contained 0.5 ml of cell suspension or 0.2 ml of crude enzyme and 0.5 ml of 1 per cent sodium alginate. The crude enzyme was obtained by precipitating the supernatant from 72-hr alginate agar-broth biphase cultures with 100 per cent $(NH_4)_2SO_4$ saturation and suspending the precipitate in 0.1 volume of potassium phosphate buffer. The reaction mixtures containing this material were covered with 1.0 ml of toluene and stoppered. All the mixtures were incubated at 37 C. At intervals the reactions in successive samples were stopped by the addition of a drop of concentrated H_2SO_4, which precipitated protein and residual alginate. This precipitate was removed by centrifugation and the supernatant analyzed for reducing sugar with the Folin-Wu reagent (1920). A standard curve was established with this reagent for sodium mannuronate; quantitative estimations of liberation of mannuronate were based on this response.

Standard manometric techniques were used to determine the ability of whole cells to oxidize mannuronate and several related substrates provided as 0.02 M solutions.

RESULTS AND DISCUSSION

Colonial and cellular morphology. After 72 hr, colonies of the isolate identified as *A. alginica* were sunken into a medium solidified with alginate, as indicated by the photograph in figure 1. These colonies bear a striking resemblance to those of the agar-digesting bacterium described recently by Swartz and Gordon (1959). Colonies of the soil isolate were less extensively alginolytic and sank only slightly.

Figure 2 presents electron micrographs of *A. alginica*. No more than one flagellum per cell was seen in any of these preparations, and each attached flagellum may be seen to orginate well within the cell. The polar placement of this organelle and the production of alginase clearly established this isolate in the genus *Alginomonas*.

The micrograph in figure 3*A* shows the polar placement of several flagella on a 6-hr cell of the unidentified pseudomonad. From one to four flagella were found on cells of this organism. The appearance of the cells in figure 3*B* demonstrates the "pitting" of many of the bacteria in cultures incubated 24 hr or longer. This phenomenon was first observed in Gram-stained preparations with the light microscope. The shadows (indicated by the arrows) confirm that the center of the cell is actually sunken and that the observation is not of an artifact.

Characterization of the isolates. The isolate identified as *A. alginica* had, in addition to properties in common with those described in *Bergey's*

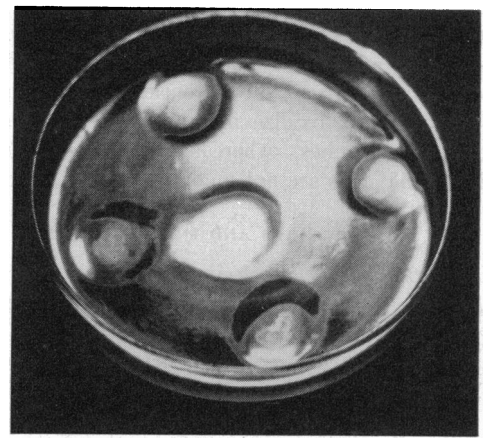

Figure 1. Appearance of colonies of *Alginomonas alginica* on a medium solidified with sodium alginate.

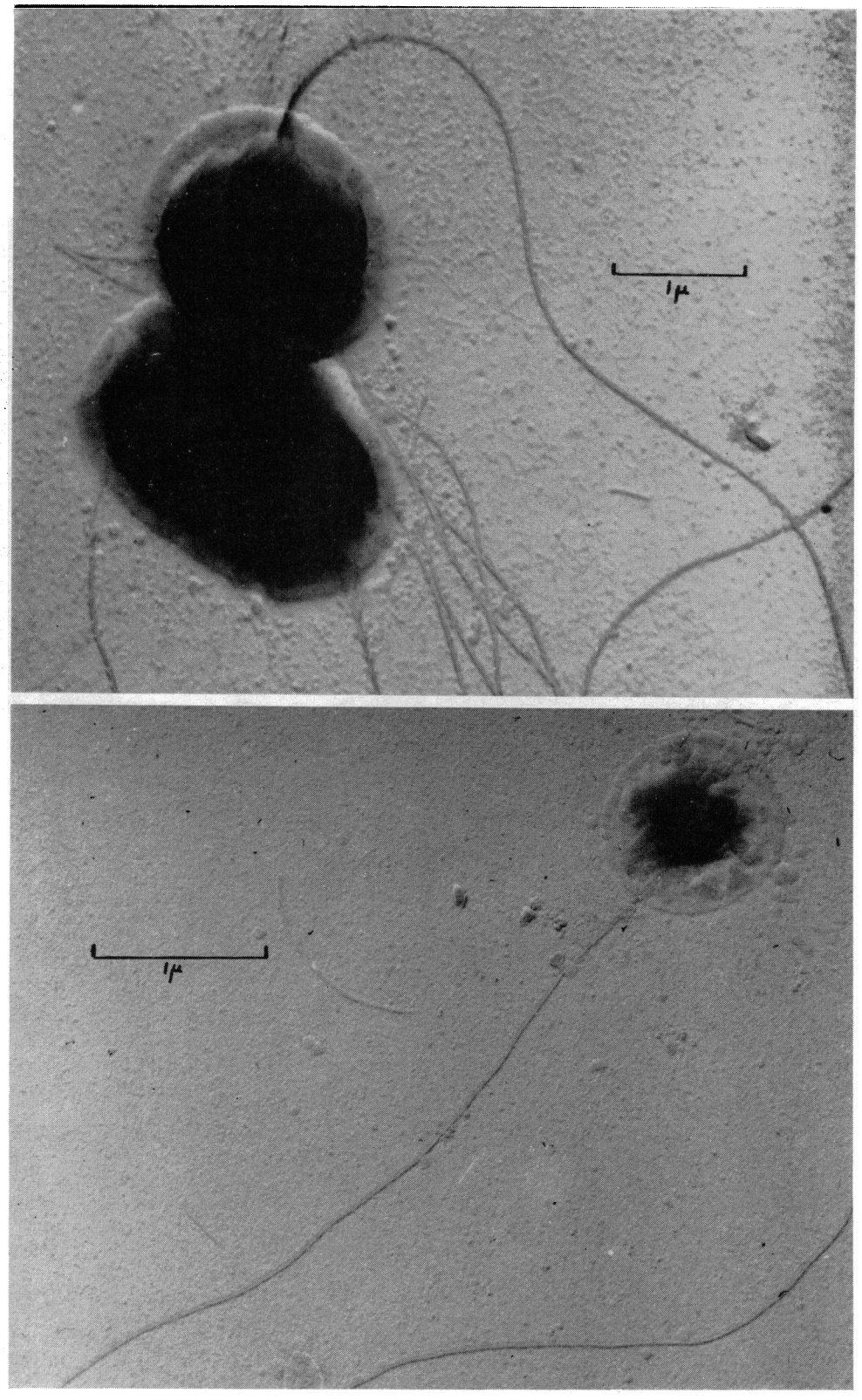

Figure 2. Electron micrographs of *Alginomonas alginica* showing polar placement of flagella.

Manual of Determinative Bacteriology (1957), the ability to produce acid but not gas from glucose, fructose, mannose, galactose, mannitol, L-arabinose, maltose, and sucrose but not from lactose, D-arabinose, xylose, glycerol, or adonitol. This additional information may be of trivial importance, however, for the species seems adequately defined without it.

In producing acid, *A. alginica* is strikingly different from our unidentified isolate, which does

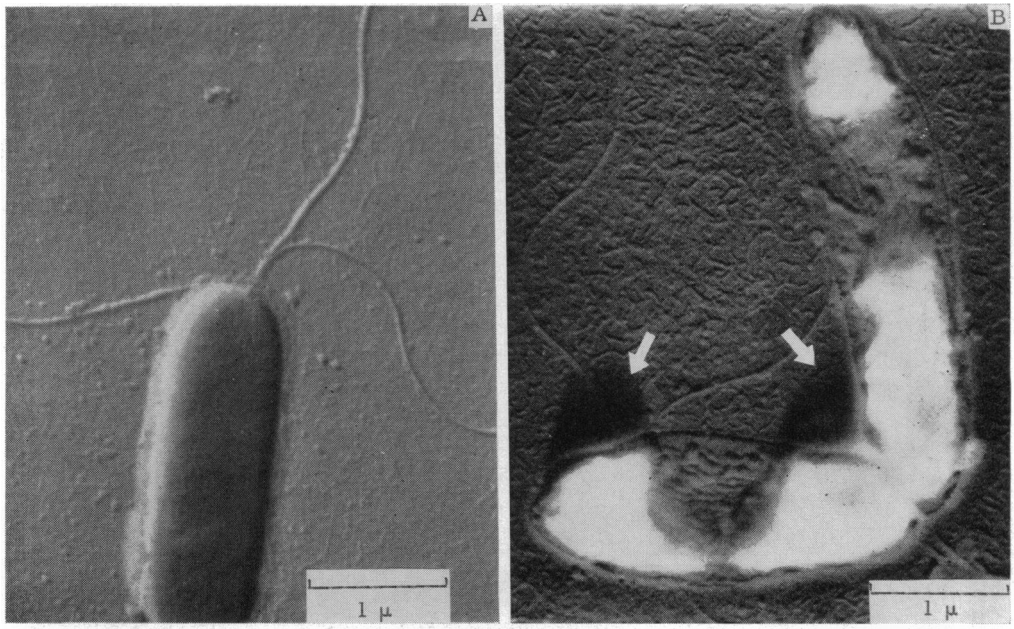

Figure 3. Electron micrographs of an unidentified alginolytic isolate from soil. *A*, cell with tuft of three flagella; *B*, "pitted" cells from 24-hr culture.

TABLE 1

Properties of an unidentified alginolytic soil isolate

Morphology	Physiology	
Cellular:	*No acid or gas in broth containing:*	Alginic acid hydrolyzed
Short, gram-negative, nonsporulating, tapering rods with 1 to 4 flagella. Average size, 0.7 by 3.4 μ. Cells "pitted" at 24 hr.	Glycerol	Agar not hydrolyzed
Colonial:	D-Arabinose	Stratiform liquefaction of gelatin
Circular, convex, slightly undulate, finely granular, opalescent, butyrous, slightly cream-colored colonies becoming buff with age on agar. Stroke cultures echinulate. Colonies yellow-brown on potato with substrate becoming dark brown. Thick pellicle on fresh or sea water broth.	L-Arabinose	Milk agar cleared
	Xylose	Starch not hydrolyzed
	Rhamnose	Cellulose not hydrolyzed
	Ducitol	H_2S not produced
	Mannitol	Nitrates not reduced to nitrites
	Sorbitol	Indole not produced
	Levulose	Urea not hydrolyzed
	Galactose	Citrates used as sole carbon source aerobically (alkaline slant)
	Glucose	Alkaline curd, slight peptonization in litmus milk
	Mannose	
	Lactose	Growth range, 4 to 37 C
	Maltose	Facultative anaerobe
	Sucrose	
	Alginic acid	

not produce acid from carbohydrates or polyols in broth. The results in table 1 indicate that this bacterium is similar to *Alginomonas nonfermentans* but differs in producing a yellow-brown pigment on potato, in failing to reduce nitrates to nitrites or to produce H_2S, and by using citrate as a sole source of carbon. It varies from the other terrestrial species, *Alginomonas terrestralginica*, in the appearance of growth on potato and action in litmus milk and from the alginolytic *Pseudomonas* species described by Humm (1946) in failing to liquefy agar or to produce acid from carbohydrates or polyols. However, the descriptive material presented in classifying the *Alginomonas* species was not extensive, and our isolate may be considered a variant of *A. terrestralginica* until more extensive taxonomic studies of this genus are made.

Alginolysis. The results in figure 4 indicate that washed cells of adapted *A. alginica* liberated mannuronate from alginate with the concentration increasing for 24 hr but decreasing sharply after that time as a result of catabolism. Nonadapted cells were not alginolytic. The exocellular enzyme from culture supernatant hydrolyzed alginate rapidly with the concentration of mannuronate reaching a maximum at 24 hr and remaining nearly constant for an additional 48 hr. Chromatographic analyses of the reaction mixtures revealed no other mobile reducing sugar than mannuronic acid.

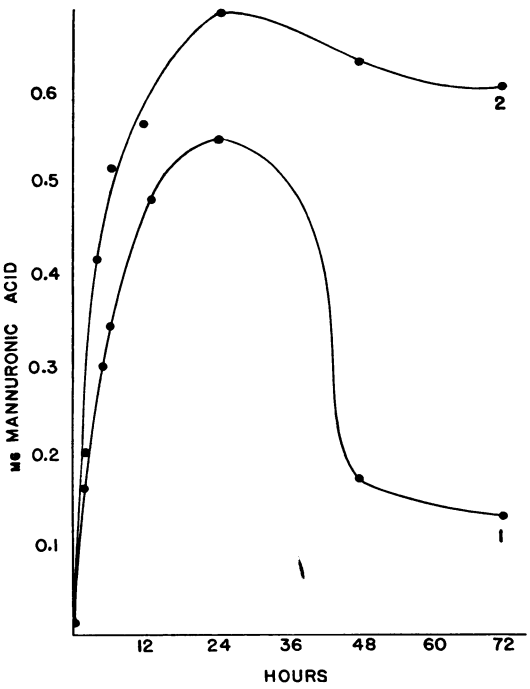

Figure 4. Alginolytic action of *Alginomonas alginica*. *1*, Adapted cells; *2*, an exocellular enzyme fraction from alginate culture supernatant.

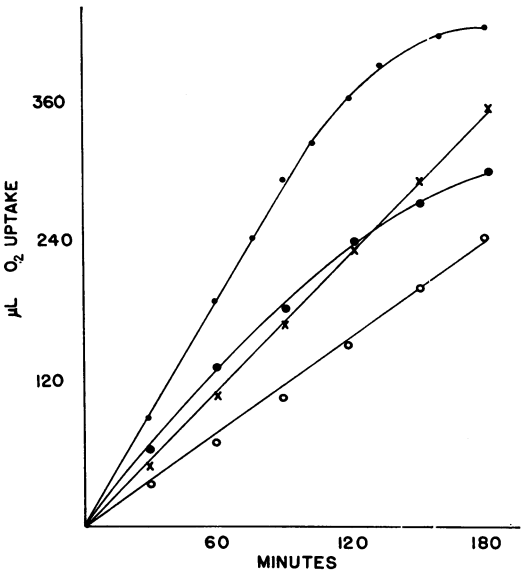

Figure 5. Oxidation of mannuronate by *Alginomonas alginica*. (○) Nonadapted cells; sodium phosphate buffer; (×) nonadapted cells, sodium-potassium phosphate buffer; (●) adapted cells, sodium phosphate buffer; (●) adapted cells, sodium-potassium phosphate buffer. Endogenous respiration (40 to 50 μL O_2/hr) subtracted.

The unidentified pseudomonad was a much less active alginolytic agent. Nonadapted cells were inactive, and presumably adapted cells slowly liberated mannuronate, with a maximal concentration of only 0.05 mg/ml being reached after 3 days of incubation and falling rapidly to zero thereafter as a result of catabolism. This correlates well with the behavior of colonies of this isolate on a medium solidified with alginate. The area surrounding the colonies did not become fluid for 3 to 4 days, and they did not sink deeply into the medium as did those of *A. alginica*.

Mannuronate oxidation. Mannuronate was oxidized without a lag by resting cells of *A. alginica* grown on either nutrient agar or alginate medium. The results in figure 5 show that adapted cells were more active than nonadapted and that the inclusion of K^+ in the suspending medium signifi-

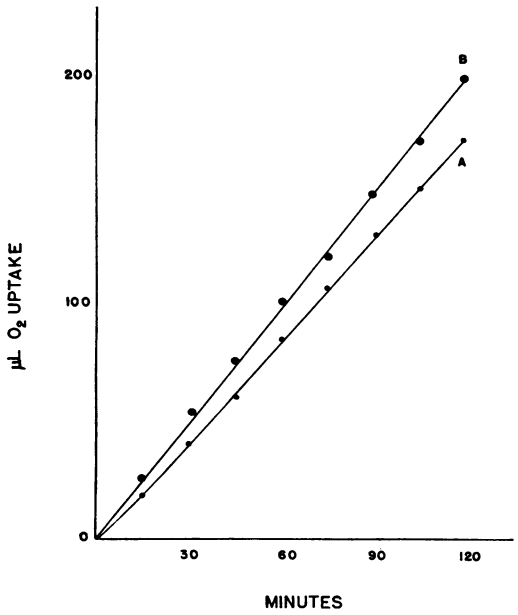

Figure 6. Oxidation of mannuronate by an unidentified alginolytic soil isolate. *A*, sodium phosphate buffer; *B*, potassium phosphate buffer. Endogenous respiration (20 to 30 μL O_2/hr) subtracted.

cantly increased the rate of oxidation. The latter phenomenon has been noted previously in the oxidation of several substrates by bacteria from the marine environment (Tomlinson and MacLeod, 1957; Payne, 1958). In addition, MacLeod *et al.* (1958) have shown that K^+ is necessary for the functioning of malic dehydrogenase in cell-free extracts of a marine bacterium.

Resting adapted and nonadapted cells of the soil isolate oxidized mannuronate about equally well. The results in figure 6 show that the inclusion of K^+ in the suspending medium did not greatly affect the rate of oxidation.

Neither of these isolates oxidized galacturonic or glucuronic acid. Nor did they oxidize D-lyxose, the pentose that results from the decarboxylation of mannuronate. Furthermore, there was no significant uptake of oxygen with L- or D-arabinose, L- or D-xylose, D-ribose, or fructuronic acid. These findings make it unlikely that decarboxylation to pentose or isomerization to keturonic acid is the initial step in the metabolism of mannuronate. Although in preliminary experiments with *A. alginica* it appeared that a mannuronate isomerase was present in cell-free extracts, this was not confirmed with more rigorous analyses.

The ability of these alginolytic bacteria to oxidize mannuronate with constitutive enzymes is a considerable departure from the findings in various laboratories with respect to the oxidation of galacturonate and glucuronate. Nearly every bacterial species which can utilize these two acids does so with induced enzymes, and induction of activity for either acid usually yields cells active with both substrates. On the other hand, the catabolism of mannuronate is accomplished by bacteria which do not have to be induced to produce uronic isomerase for the first dissimilative step but are capable of producing enzymes specific for the dissimilation of the mannuronic isomer. Bacteria with this latter property are quite rare.

An apparent ecological anomaly is represented by our unidentified soil isolate. It is possible that the ability to grow in sea water medium reflects a marine origin for the organism in the not too distant past. Furthermore, the abilities to hydrolyze alginate and to oxidize mannuronate are remarkable, since these substrates have been reported to occur only in marine algae and not in inland soil. The presence of either capability without the other might be explained by nonspecificity, but the possession of both lytic and oxidative properties would require speculation that other sources of alginate may occur in nature, if we do not assume a marine origin for the isolate.

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SUMMARY

Alginolytic bacteria which oxidize mannuronic acid have been isolated from brown algae and from soil enriched with alginic acid. *Alginomonas alginica* was obtained from sunken colonies on a medium solidified with sodium alginate and inoculated with ground brown algae. Adapted cells rapidly liberated mannuronate when incubated with sodium alginate. Maximal concentration was reached in 24 hr and decreased rapidly thereafter as a result of catabolism. An exocellular alginase was present in alginate agar-broth biphase culture supernatant.

The unidentified isolate was more weakly alginolytic. The colonies did not sink appreciably,

nor did the organism produce detectable exocellular alginase. Adapted cells liberated mannuronate very slowly. Both *A. alginica* and the unidentified isolate oxidized mannuronate constitutively, but did not oxidize galacturonate or glucuronate.

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