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# Two Types of Bacterial Alginate Lyases

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**The extracellular alginate lyases were purified from *Vibrio harveyi* AL-128 and *V. alginolyticus* ATCC 17749. The former enzyme appears to be specific for α-1,4 bonds involving L-guluronate units in alginate, whereas the latter exhibits specificity for β-1,4 bonds involving D-mannuronate units. The molecular weights of the enzymes were estimated to be 57,000 and 47,000, and they had isoelectric points of 4.3 and 4.6, respectively. The enzyme from strain AL-128 was most active at NaCl concentrations of 0.3 to 1.0 M. Optimum activity of the enzyme from strain ATCC 17749 was found in the presence of 5 to 10 mM $CaCl_2$.**

Alginic acid is a commercially important algal polysaccharide. This copolymer is formed from (1→4)-linked α-L-guluronic acid and (1→4)-linked β-D-mannuronic acid units. The residues are arranged in irregular blocks along the linear chain (5). Alginic acid is variable in D-mannuronic acid and L-guluronic acid content, and both mannuronic acid-rich and guluronic acid-rich fractions can be prepared by salt fractionation procedures (7, 8).

Alginate-degrading enzyme, alginate lyase (EC 4.2.2.3), has been found in a variety of sources, including marine molluscs, bacteria, and brown algae (2, 15). Alginate lyases isolated from molluscs generally show a preference for a mannuronate-rich substrate (20, 21), and a purified molluscan enzyme was characterized as an endomannuronide lyase (4). A bacterial intracellular enzyme preferred a guluronate-rich substrate (19), whereas several bacterial extracellular enzymes showed a preference for either mannuronate or guluronate blocks in the alginate molecules (2, 3, 6, 7, 22). With a few exceptions (22, 24), bacterial alginate lyases have been examined as either crude or partially purified preparations.

Our previous report showed that *Vibrio* sp. strain AL-128 and *Vibrio alginolyticus* ATCC 17749 have the capacity to degrade alginate (13). We tried to obtain different types of alginate lyases from bacteria in purified form and to examine further the relationship between these enzymes and their substrates. The present report documents the purification and characterization of extracellular alginate lyases from *Vibrio* sp. AL-128 and *V. alginolyticus* ATCC 17749.

## MATERIALS AND METHODS

**Sodium alginate, M and G blocks, and chemicals.** Sodium alginate (lot KPH7033) was obtained from Wako Pure Chemical Industries Ltd. The mannuronate-guluronate ratio was 1.28. Oligomers enriched with sodium mannuronate (M-rich fraction) and sodium guluronate (G-rich fraction) were prepared from the commercial alginate after partial acid hydrolysis by the method of Haug et al. (9). The M-rich fraction was then treated with the purified enzyme from strain AL-128 to remove L-guluronate units. The solution was made to 66% alcohol, and the M-rich precipitate was then washed with alcohol and dried. The G-rich fraction was similarly prepared with the purified enzyme from strain ATCC 17749. The M-rich and G-rich preparations were used throughout our experiments and are defined as M blocks and G blocks, respectively. The average degrees of polymerization of sodium alginate, M blocks, and G blocks were estimated to be 55, 13, and 9 saccharide, respectively, by the periodate oxidation method and the phenol-sulfuric acid method (10, 11). D-Mannuronic acid lactone was obtained from Sigma Chemical Co., St. Louis, Mo., and a small amount of 0.1 N sodium hydroxide was added to split lactone. L-Guluronic acid was prepared from the hydrolysate of G blocks. Sephadex G-100, phenyl Sepharose CL-4B, and Blue Sepharose CL-6B were obtained from Pharmacia LKB Biotechnology. All other chemicals were obtained from commercial sources.

**$^{13}$C-NMR spectra of G blocks and M blocks.** $^{13}$C-nuclear magnetic resonance ($^{13}$C-NMR) spectra of G blocks and M blocks were obtained from samples dissolved in $D_2O$ (oligomer concentration, 40 mg/0.6 ml). Chemical shifts were expressed in parts per million relative to external 3-(trimethylsilyl)-1-propanesulfonic acid. $^{13}$C-NMR spectra were recorded on a JEOL GX-400 (100 MHz) spectrometer. Experimental parameters included 32,000 datum points, a spectral width of 22 kHz, a 45° pulse, a pulse repetition time of 3.0 s, and 16,384 scans.

**Bacterial strains and culture fluid.** Alginate lyase-producing strain AL-128 was isolated from seawater in Japan (13). *V. alginolyticus* ATCC 17749, the type strain of the species (1), was obtained from the American Type Culture Collection. These strains were maintained as slant cultures. The liquid medium (pH 7.8) used to culture the organisms contained 1.0% peptone, 0.1% yeast extract, 3.0% NaCl, and 0.5% sodium alginate. Both strains were grown in the following manner. The cells from a slant culture were inoculated into 20-ml aliquots of liquid medium in 50-ml flasks and incubated without shaking at 25°C for 2 days. Each culture was then transferred to 1,000 ml of liquid medium in a 3,000-ml flask and incubated at 25°C for 5 days. The clarified culture fluid was obtained by centrifugation of the cell suspension at 10,000 × *g* for 30 min.

**Enzyme purification.** All chromatographic operations were carried out at 5°C and a flow rate of 26 ml/h.

**(i) Ammonium sulfate fractionation.** The culture fluid of each strain was adjusted to 75% saturation with solid ammonium sulfate and allowed to stand overnight. The precipitate formed was collected by centrifugation and dissolved in 150 (strain AL-128) or 200 (strain ATCC 17749) ml of 10 mM sodium phosphate buffer (pH 6.8) for further purification.

**(ii) Column chromatography of the ammonium sulfate fraction from strain AL-128.** The enzyme solution (40 ml)

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was loaded onto a phenyl Sepharose CL-4B column (2.0 by 24 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.8) containing 1.8 M ammonium sulfate. After being washed with 300 ml of the buffer, alginate lyase was eluted by application of a decreasing linear gradient of 450 ml from 1.8 to 0 M ammonium sulfate in the same buffer. The active fractions (see tubes 112 to 120 in Fig. 2) were pooled and concentrated with polyethylene glycol. The prepared solution was dialyzed against 50 mM Tris-HCl buffer (pH 8.0) and used as the purified enzyme. Purified alginate lyase was stored at −20°C.

**(iii) Column chromatography of the ammonium sulfate fraction from strain ATCC 17749.** The enzyme solution (50 ml) was passed through a phenyl Sepharose CL-4B column by the procedure described above. The active fractions (see tube 160 to 178 in Fig. 4) were pooled, concentrated with polyethylene glycol, and dialyzed against 50 mM sodium acetate buffer (pH 6.0). Half of the dialyzed preparation was applied to a Blue Sepharose CL-6B column (2.5 by 17 cm) equilibrated with the same buffer. After being washed with 180 ml of the same buffer, alginate lyase was eluted with an increasing NaCl gradient (0 to 1 M) in 300 ml of the same buffer. The fractions containing enzyme activity (see 115 to 123 in Fig. 5) were pooled and concentrated with polyethylene glycol. The sample was dialyzed against 50 mM Tris-HCl buffer (pH 8.0), and the purified alginate lyase was stored at −20°C.

**Molecular weight determination.** The molecular weight of the purified enzyme was determined by sucrose density gradient centrifugation (18) at 178,900 × *g* and 8°C for 20 h with a Hitachi RPS65T rotor. RNase A (13,700), chymotrypsinogen A (25,000), ovalbumin (43,000), and bovine serum albumin (67,000) were used as markers.

**Isoelectric point determination.** Isoelectric point were determined by analytical isoelectric focusing using Ampholine PAG plates (pHs 4.0 to 6.5, 110 by 245 mm; Pharmacia LKB) with Coomassie brilliant blue R-250 as the protein stain. The LKB pI markers were amyloglucosidase (3.65), acetylated cytochrome *c* (3.95), glucose oxidase (4.25), c. phycocyanin (4.75 and 4.85), β-lactoglobulin a (5.25), β-lactoglobulin b (5.35), and azurin (5.65). Enzyme activity in the gel was determined by sectioning the gel into 0.5-cm slices which were incubated overnight with 0.3 ml of alginate solution under standard conditions. The extracts were then assayed for unsaturated uronates.

**$K_m$ determination.** The $K_m$ was determined by the Lineweaver-Burk method (14). A 0.1-ml volume of a 0.5-U/ml enzyme solution was incubated with 0.3-ml volumes of substrate solutions (G blocks or M blocks) of different concentrations at 37°C for 1 min.

**Thin-layer chromatography.** Thin-layer chromatography of oligosaccharides was performed on a silica gel 60 plastic sheet (Merck & Co. Inc., Darmstadt, Germany) in a solvent containing *n*-butanol–acetic acid–water (5:2:3 [vol/vol]). The saccharides were visualized by spraying the plate with diphenylamine-aniline-phosphate reagent.

**Enzyme assays. (i) Reducing group assay.** To 1.5 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 0.4% sodium alginate and 0.4 M NaCl was added 0.5 ml of the enzyme solution containing 20 mM $CaCl_2$. After incubation at 37°C for 20 min, the reaction was stopped by adding 2 ml of the alkaline copper reagent and the reducing sugar produced was determined by the Somogyi procedure (23). One unit of enzyme activity was defined as the amount which liberated 1 μmol of D-mannuronic or L-guluronic acid per min under the above-described conditions.

**(ii) Thiobarbituric acid assay.** To 0.3 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 0.4% sodium alginate and 0.4 M NaCl was added 0.1 ml of the enzyme solution containing 20 mM $CaCl_2$. After incubation at 37°C for 20 min, 0.5 ml of 0.025 N $HIO_4$ in 0.125 N $H_2SO_4$ was added to the reaction mixture. The formyl pyruvate produced was determined by measuring the $A_{548}$ of the mixture after adding the thiobarbituric acid reagent (26).

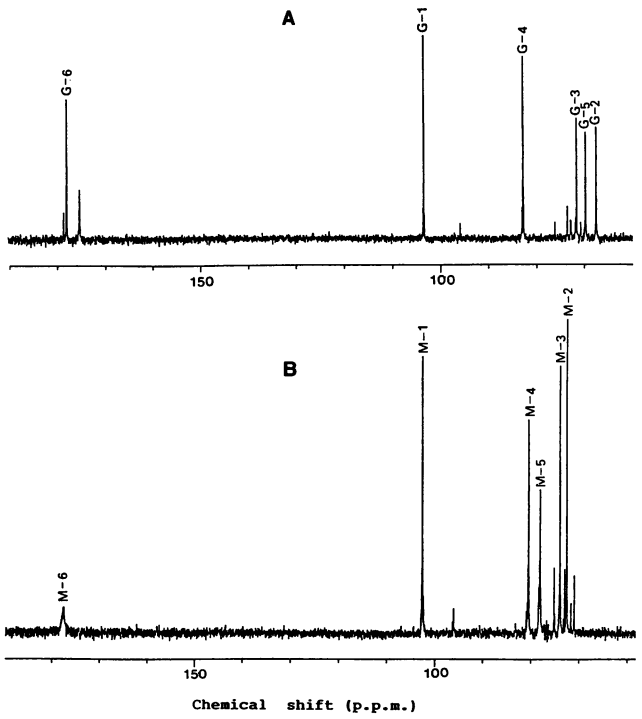


FIG. 1. $^{13}$C-NMR spectra (100 MHz) of solutions (40 mg/0.6 ml) in $D_2O$ at 23°C of sodium salts of G blocks (A) and M blocks (B).

**(iii) Protein determination.** Protein was determined either by the method of Lowry et al. (16) with bovine serum albumin as the standard or by determination of the $A_{280}$.

**Classification of strain AL-128.** Identification of strain AL-128 was done by the criteria described in *Bergey's Manual of Systematic Bacteriology* (1).

## RESULTS

**$^{13}$C-NMR spectra of G blocks and M blocks.** The 100-MHz $^{13}$C-NMR spectra of the prepared G blocks and M blocks used as substrates are presented in Fig. 1. The G blocks and M blocks each exhibited six dominant peaks. By reference to the previously described spectra of alginates having different guluronic and mannuronic acid contents (5, 17), the major peaks in Fig. 1 confirmed the high content of guluronic and mannuronic acids in the two blocks, respectively.

**Alginate lyase from *V. harveyi* AL-128. (i) Purification of the enzyme.** Purification procedures are summarized in Table 1. The elution profiles for alginate lyase and protein from the phenyl Sepharose CL-4B column (Fig. 2) revealed only one enzymatically active protein peak. Alginate lyase from strain AL-128 was purified more than 70-fold in a yield of 32.3% and used throughout the experiments.

**(ii) General properties of the enzyme.** The isoelectric point of the enzyme determined by isoelectric focusing was pH 4.3, and the enzyme activity was detected over a similar pH

TABLE 1. Purification of alginate lyase from the culture fluid of *V. harveyi* AL-128 and that of *V. alginolyticus* ATCC 17749

| Organism (amt of culture fluid [ml]) | Procedure | Total protein (mg) | Total activity (U) | Sp act (U/mg) | Yield (%) | Purification (fold) | Optical density at 548 nm[a] | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | G | M |
| *V. harveyi* AL-128 (1,870) | Culture fluid | 9,530 | 248 | 0.026 | 100 | 1.00 | 0.75 | 0.05 |
| | $(NH_4)_2SO_4$ precipitation | 505 | 175 | 0.347 | 70.6 | 13.3 | 0.80 | 0.03 |
| | Phenyl Sepharose CL-4B | 42.3 | 80.0 | 1.89 | 32.3 | 72.7 | 0.82 | 0.00 |
| *V. alginolyticus* ATCC 17749 (2,700) | Culture fluid | 10,400 | 113 | 0.011 | 100 | 1.00 | 0.40 | 0.48 |
| | $(NH_4)_2SO_4$ precipitation | 759 | 79.5 | 0.104 | 70.0 | 9.45 | 0.35 | 0.50 |
| | Phenyl Sepharose CL-4B | 161 | 60.0 | 0.373 | 53.1 | 33.9 | 0.03 | 0.76 |
| | Blue Sepharose CL-6B | 22.2 | 42.8 | 1.93 | 37.9 | 175 | 0.00 | 0.80 |

[a] The enzyme activity of alginate lyase (0.05 U/ml) toward G blocks or M blocks was determined by the thiobarbituric acid assay method.

range (Fig. 3). The estimated molecular weight was 57,000. The $K_m$ was $7.4 \times 10^{-3}$%, which approximates to $5.1 \times 10^{-5}$ M, assuming an average degree of polymerization of 9 for the G blocks.

The optimal pH of the enzyme was determined with 50 mM sodium acetate (pH 3.5 to 6.0), morpholineethanesulfonic acid-NaOH (pH 5.0 to 8.0), Tris-HCl (pH 7.5 to 9.0), and $NH_3$-$NH_4Cl$ (pH 8.5 to 11.0) buffers in the assay system. The enzyme exhibited maximal activity at pH 7.8. The stability of the enzyme at various pHs was examined by incubating it in the above buffers and glycine (Na)-NaOH (pH 11.0 to 13.0) buffer at 5°C for 20 h. The enzyme was stable in the pH range from 6.0 to 11.0.

The effects of increasing concentrations of the chlorides of $Na^+$, $Mg^{2+}$, and $Ca^{2+}$ on the enzyme activity toward sodium alginate were investigated (Table 2). $Na^+$ was the most potent effector, and enzyme activity was maximal (24 times greater than the control) at concentrations of 0.3 to 1.0 M NaCl. $Mg^{2+}$ and $Ca^{2+}$ (at 1 mM) caused some activation of the enzyme, but the enzyme was almost completely inhibited by $Zn^{2+}$ and $Hg^{2+}$. $Ag^+$, $Cu^{2+}$, $Mn^{2+}$, $Pb^{2+}$, $Fe^{3+}$, and EDTA gave about 20 to 70% inhibition at the same concentration.

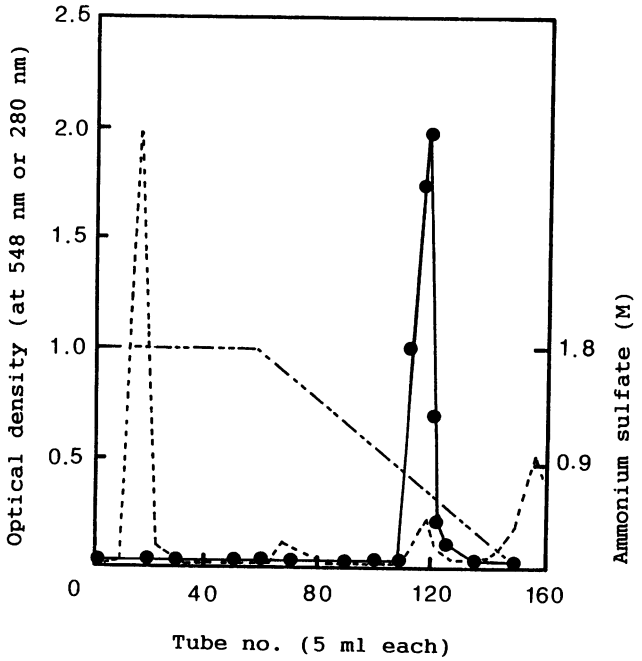


FIG. 2. Chromatography of alginate lyase from *V. harveyi* AL-128 on a phenyl Sepharose CL-4B column. Symbols: ●, enzyme activity; – – – –, $A_{280}$; ··——··, concentration of ammonium sulfate.

**Alginate lyase from *V. alginolyticus* ATCC 17749. (i) Purification of the enzyme.** Purification procedures are summarized in Table 1. The elution profiles for alginate lyase and protein from phenyl Sepharose CL-4B are shown in Fig. 4. Two enzymatically active peaks were detected. The first passed through the column, whereas the second was adsorbed on the column and subsequently eluted with a gradually decreasing ammonium sulfate gradient. Further purification of the first fractions was hampered by the presence of a large amount of contaminating proteins. Fractions from the second peak (166 to 174) were pooled, concentrated with polyethylene glycol, and further purified by Blue Sepharose CL-6B column chromatography (Fig. 5). Alginate lyase from strain ATCC 17749 was purified more than 170-fold in a yield of 37.9% and used throughout the experiments.

**(ii) General properties of the enzyme.** The isoelectric point of the enzyme was 4.6, and the enzyme activity was detected over a similar pH range (Fig. 3). The molecular weight was

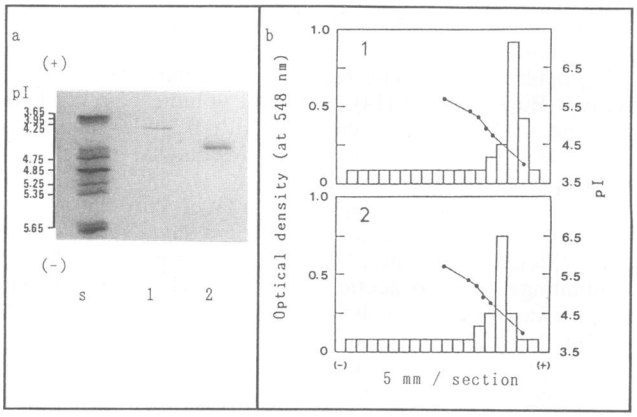


FIG. 3. Isoelectric focusing of purified alginate lyases from *V. harveyi* AL-128 and *V. alginolyticus* ATCC 17749. (a) Horizontal isoelectric focusing polyacrylamide gel electrophoresis was used to analyze the enzymes as described in Materials and Methods. Lanes: s, Pharmacia pI calibration kit; 1, strain AL-128; 2, strain ATCC 17749. (b) Panels: 1, a lane containing the purified enzyme from strain AL-128 was sliced, and enzyme activity was detected as described in Materials and Methods; 2, the purified enzyme from strain ATCC 17749.

TABLE 2. Effects of metal ions on the activities of alginate lyases from *V. harveyi* AL-128 and *V. alginolyticus* ATCC 17749

| Chloride concn (M) | Relative enzyme activities[a] | | |
|---|---|---|---|
| | NaCl | $MgCl_2$ | $CaCl_2$ |
| None (control) | 100, 100 | 100, 100 | 100, 100 |
| $1 \times 10^{-5}$ | 116, 100 | 217, 100 | 167, 117 |
| $1 \times 10^{-3}$ | 183, 117 | 283, 117 | 250, 1,770 |
| $1 \times 10^{-2}$ | 217, 117 | 417, 357 | 350, 3,300 |
| $1 \times 10^{-1}$ | 667, 150 | 717, 300 | 233, 817 |
| $3 \times 10^{-1}$ | 2,380, 350 | —[b] | — |
| $1 \times 10^{0}$ | 2,400, 367 | 100, 117 | 92, 167 |
| $2 \times 10^{0}$ | 617, 100 | — | — |

[a] Alginate lyase activity was determined by the thiobarbituric acid assay method as described in Materials and Methods. The paired values shown are for strains AL-128 and ATCC 17749, respectively.
[b] —, not determined.

47,000. The $K_m$ of the enzyme for M blocks was $11.1 \times 10^{-3}$%, and this value was equivalent to $5.4 \times 10^{-5}$ M when an average degree of polymerization of 13 for the M blocks was used. The enzyme exhibited optimal activity at pH 8.2 and was stable within the pH range of 6.0 to 11.0.

$Ca^{2+}$ had the largest stimulatory effect on the enzyme (activity 33 times greater than that of the control) at concentrations of 5 to 10 mM, whereas $Na^+$ at the same concentration had no significant effect on enzyme activity (Table 2). The enzyme was completely inhibited by $Zn^{2+}$ and EDTA at a concentration of 1 mM, while $Ag^+$, $Cu^{2+}$, $Hg^{2+}$, and $Fe^{3+}$ gave about 30 to 80% inhibition at the same concentration.

**Substrate specificities of alginate lyases from strains AL-128 and ATCC 17749.** The specificities of the enzymes toward G- and M-block substrates were studied at each purification step (Table 1). In the culture fluid from strain AL-128, an almost specific action toward G blocks was found. On the other hand, there were more than two alginate lyase components with different activities in the culture fluid of strain ATCC 17749. The minor component fractions of alginate

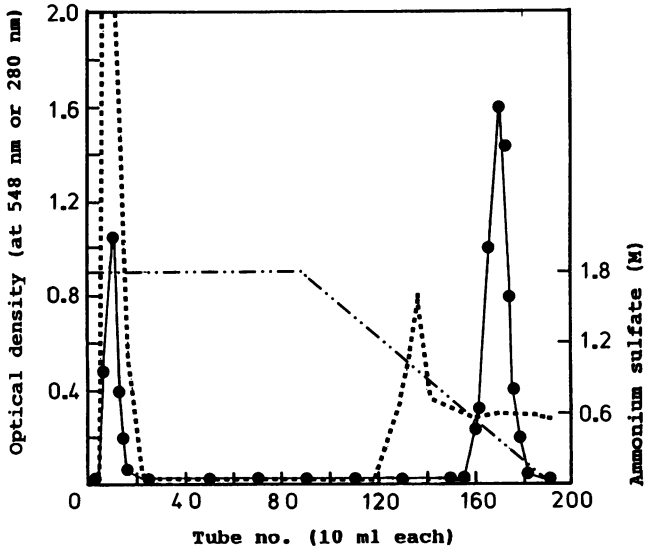


FIG. 4. Chromatography of alginate lyase from *V. alginolyticus* ATCC 17749 on a phenyl Sepharose CL-4B column. Symbols: ●, enzyme activity; ----, $A_{280}$; "——", concentration of ammonium sulfate.

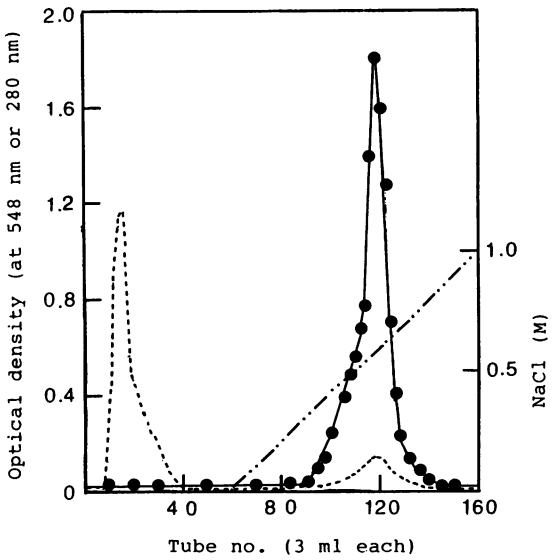


FIG. 5. Chromatography of alginate lyase from *V. alginolyticus* ATCC 17749 on a Blue Sepharose CL-6B column. Symbols: ●, enzyme activity; ----, $A_{280}$; "——", concentration of sodium chloride.

lyases from strain ATCC 17749, which passed through the phenyl Sepharose CL-4B column, were active toward both G and M blocks. The enzyme adsorbed on the same column was specific only for M blocks.

Each 1.0 ml of enzyme solution from strains AL-128 (0.01 U) and ATCC 17749 (0.013 U) was incubated with either G or M blocks (40 mg of each) in the presence of 1 mM $CaCl_2$ for 24 h. Thin-layer chromatography was carried out to detect reaction products. The enzyme from strain AL-128 attacked G blocks to give a monosaccharide and a series of oligosaccharides, but it did not attack M blocks (Fig. 6). By contrast, the enzyme from strain ATCC 17749 was active only on M blocks and produced both a monosaccharide and a series of oligosaccharides (Fig. 6). Data indicate that the purified enzyme from strain AL-128 may be characterized as a poly(1,4-α-L-guluronide) lyase, while that from strain ATCC 17749 may be designated a poly(1,4-β-D-mannuronide) lyase.

**Classification of strain AL-128.** *Vibrio* sp. strain AL-128 displayed the following characteristics: straight rod synthesizing lateral flagella on solid medium, positive for growth at 35°C and utilization of D-mannose, cellobiose, D-gluconate, D-glucuronate, heptanoate, α-ketoglutarate, L-serine, L-glutamate, and L-tyrosine; negative for arginine dihydrolase, acetoin and/or diacetyl production and utilization of β-hydroxybutyrate, D-sorbitol, ethanol, L-leucine, γ-aminobutyrate, and putrescine. The G+C content of the DNA is 48 mol%. On the basis of the criteria given in *Bergey's Manual of Systematic Bacteriology*, the strain was assigned to the species *V. harveyi*.

## DISCUSSION

In many studies, NaCl has been reported as an important activator of bacterial alginate lyases (19, 22, 25), and a few studies have dealt with $CaCl_2$. The promoting effects of high salt concentrations on the enzyme activity may be due in part to removal of bound water from sodium alginate molecules or possibly to the effects of charge in forming the enzyme-alginate complex (12). Toward the enzymes from strains AL-128 and ATCC 17749, $Na^+$, $Mg^{2+}$, and $Ca^{2+}$ had

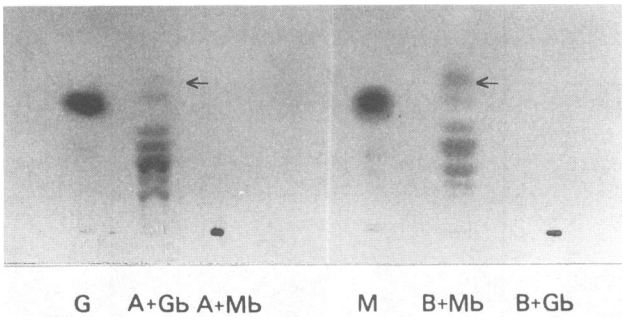


FIG. 6. Thin-layer chromatography of degradation products from G and M blocks. Lanes: G, sodium L-guluronate; A+Gb, reaction mixture of enzyme from *V. harveyi* AL-128 and G blocks; A+Mb, reaction mixture of enzyme from *V. harveyi* AL-128 and M blocks; M, sodium D-mannuronate; B+Mb, reaction mixture of enzyme from *V. alginolyticus* ATCC 17749 and M blocks; B+Gb, reaction mixture of enzyme from *V. alginolyticus* ATCC 17749 and G blocks. The arrows indicate the position of the component which moved more rapidly than L-guluronate or D-mannuronate.

promoting effects and maximal activity appeared in the presence of 0.3 to 1.0, $1 \times 10^{-1}$, and 0.5 to 1.0 $\times 10^{-2}$ M concentrations of the ions, respectively. The concentrations of NaCl for activating alginate lyases in prior descriptions were lower than this value. We investigated the effects of metal ions on the enzyme by using sodium alginates from different commercial sources (one from Wako Chemical Co., two with high and low viscosities from Sigma Chemical Co., and one from Ishizu pharmaceutical Co., Japan) and M and G blocks. With all of these substrates, $Na^+$ had the largest stimulatory effect and $Ca^{2+}$ had a slight one on the activity of the enzyme from strain ATCC 17749. The finding that both enzymes were activated by KCl at the same concentration as NaCl suggests that the metal ions promote activation of the bacterial alginate lyases.

On each thin-layer chromatogram of the reaction products, one component moved more rapidly than the D-mannuronate or L-guluronate standard. The component exhibited either a weak grayness (Fig. 6, A+Gb) or a pinkness (Fig. 6, B+Mb), whereas other products were black when sprayed with the diphenylamine-aniline-phosphate reagent. The rapidly moving component may represent a secondary product derived from an unstable intermediate. The more slowly moving components indicated a monosaccharide and a series of unsaturated oligosaccharides. The disaccharides have been reported as products of substrate breakdown by bacterial alginate lyases (2, 16, 22, 24), but few enzymes have been reported to degrade substrates to monosaccharide products (20).

Production of alginate lyases is one of the characteristics used to differentiate species of the genus *Vibrio* (1). According to the description given in *Bergey's Manual of Systematic Bacteriology*, production of an extracellular alginate-degrading enzyme is negative for *V. alginolyticus*. In this and a previous study (13), strain ATCC 17749 was found to release a considerable amount of alginate lyases into the culture fluid. From these observations the question arises as to whether production of alginase is a suitable characteristic for differentiation of *Vibrio* species. We suggest that it be excluded from the characteristics of *Vibrio* species.

## REFERENCES

1. **Baumann, P., A. L. Furniss, and J. V. Lee.** 1984. Genus I. *Vibrio* Pacini 1854, 411$^{AL}$, p. 518–538. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
2. **Boyd, J., and J. R. Turvey.** 1977. Isolation of a poly-α-L-guluronate lyase from *Klebsiella aerogenes*. Carbohydr. Res. **57**:163–171.
3. **Doubet, R. S., and R. S. Quatrano.** 1982. Isolation of marine bacteria capable of producing specific lyases for alginate degradation. Appl. Environ. Microbiol. **44**:754–756.
4. **Elyakova, L. A., and V. V. Favorov.** 1974. Isolation and certain properties of alginate lyase VI from the mollusk *Littorina* sp. Biochim. Biophys. Acta **358**:341–354.
5. **Grasdalen, H., B. Larsen, and O. Smidsrod.** 1981. $^{13}$C-N.M.R. studies of monomeric composition and sequence in alginate. Carbohydr. Res. **89**:179–181.
6. **Hansen, J. B., R. S. Doubet, and J. Ram.** 1984. Alginase enzyme production by *Bacillus circulans*. Appl. Environ. Microbiol. **47**:704–709.
7. **Haug, A.** 1959. Fractionation of alginic acid. Acta Chem. Scand. **13**:601–603.
8. **Haug, A.** 1959. Ion exchange properties of alginate fractions. Acta Chem. Scand. **13**:1250–1251.
9. **Haug, A., B. Larsen, and O. Smidsrod.** 1966. A study of the constitution of alginic acid by partial acid hydrolysis. Acta Chem. Scand. **20**:183–190.
10. **Hay, G. W., B. A. Lawis, and F. Smith.** 1965. Periodate oxidation of polysaccharides: general procedures. Methods Carbohydr. Chem. **5**:357–361.
11. **Hodge, J. E., and B. T. Hofreiter.** 1962. Phenol-sulfuric acid colorimetric method. Methods Carbohydr. Chem. **1**:388–389.
12. **Jacobson, B.** 1955. On the interpretation of dielectric constants of aqueous macromolecular solutions. J. Am. Chem. Soc. **77**:2919–2926.
13. **Kitamikado, M., K. Yamaguchi, C. H. Tseng, and B. Okabe.** 1990. Method designed to detect alginate-degrading bacteria. Appl. Environ. Microbiol. **56**:2939–2940.
14. **Lineweaver, H., and D. Burk.** 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56**:658–666.
15. **Linker, A., and L. R. Evans.** 1984. Isolation and characterization of an alginase from mucoid strains of *Pseudomonas aeruginosa*. J. Bacteriol. **159**:958–964.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265–275.
17. **Mackie, W.** 1983. Aspects of the conformation of polyguluronate in the solid state and in solution. Int. J. Biol. Macromol. **5**:329–341.
18. **Martin, R. G., and B. N. Ames.** 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. **236**:1372–1379.
19. **Min, K. H., S. F. Sasaki, Y. Kashiwabara, H. Suzuki, and K. Nisizawa.** 1977. Multiple components of endo-polyguluronide lyase of *Pseudomonas* sp. J. Biochem. **81**:539–546.
20. **Nakada, H. I., and P. C. Sweeny.** 1967. Alginic acid degradation by eliminase from abalone hepatopancreas. J. Biol. Chem. **242**:845–851.
21. **Nisizawa, K., S. Fujibayashi, and Y. Kasiwabara.** 1968. Alginate lyases in the hepatopancreas of a marine mollusc, *Dolabella auricula* Solander. J. Biochem. **64**:25–37.
22. **Remeo, T., and J. F. Preston.** 1986. Purification and structural properties of an extracellular (1-4)-β-D-mannuronan-specific alginate lyase from a marine bacterium. Biochemistry **25**:8385–8391.
23. **Somogyi, M.** 1952. Notes on sugar determination. J. Biol. Chem. **195**:19–23.
24. **Stevens, R. A., and R. E. Levin.** 1977. Purification and characteristics of an alginase from *Alginovibrio aquatilis*. Appl. Environ. Microbiol. **3**:1156–1161.
25. **Waksman, S. A., and M. C. Allen.** 1934. Decomposition of polyuronides by fungi and bacteria. J. Am. Chem. Soc. **56**:2701–2705.
26. **Weissbach, A., and J. Hurwitz.** 1959. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. J. Biol. Chem. **234**:705–709.