JOURNAL OF BACTERIOLOGY, Mar. 1999, p. 1409–1414 Vol. 181, No. 5
0021-9193/99/$04.00+0

Cloning and Expression of the *algL* Gene, Encoding the *Azotobacter chroococcum* Alginate Lyase: Purification and Characterization of the Enzyme

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Received 30 July 1998/Accepted 15 December 1998

The alginate lyase-encoding gene (*algL*) of *Azotobacter chroococcum* was localized to a 3.1-kb *Eco*RI DNA fragment that revealed an open reading frame of 1,116 bp. This open reading frame encodes a protein of 42.98 kDa, in agreement with the value previously reported by us for this protein. The deduced protein has a potential N-terminal signal peptide that is consistent with its proposed periplasmic location. The analysis of the deduced amino acid sequence indicated that the gene sequence has a high homology (90% identity) to the *Azotobacter vinelandii* gene sequence, which has very recently been deposited in the GenBank database, and that it has 64% identity to the *Pseudomonas aeruginosa* gene sequence but that it has rather low homology (15 to 22% identity) to the gene sequences encoding alginate lyase in other bacteria. The *A. chroococcum* AlgL protein was overproduced in *Escherichia coli* and purified to electrophoretic homogeneity in a two-step chromatography procedure on hydroxyapatite and phenyl-Sepharose. The kinetic and molecular parameters of the recombinant alginate lyase are similar to those found for the native enzyme.

Alginates are linear polysaccharides composed of (1,4)-linked β-D-mannuronic acid and its C-5 epimer, α-L-guluronic acid. These uronic acids are arranged in block structures which may be homopolymeric (polymannuronic acid or polyguluronic acid) or heteropolymeric random sequence (15). The proportion and arrangement of the block structures vary greatly in alginates from different sources and determine the physical properties of the polymer, particularly the ability to form gels in the presence of divalent cations (21). Alginates are synthesized as cell wall components by brown seaweeds and as exopolysaccharides by two families of heterotrophic bacteria, *Pseudomonadaceae* and *Azotobacteriaceae*. Marine alga alginate is used widely in the food, pharmaceutical, textile, and oil industries. Bacterial alginate is an acetylated polymer of D-mannuronic and L-guluronic acids, and evidence has been presented for the location of *O*-acetyl groups at the 2 and/or 3 position of D-mannuronosyl residues (41). In their pioneering work, Lawson and Stacey (26) described the existence of two capsular polysaccharides in *Azotobacter chroococcum*, and, more recently, one of these exocellular polysaccharides was identified as an alginate (9). In cystic fibrosis patients, *Pseudomonas aeruginosa* produces alginate, which facilitates the attachment of the bacterium to tracheal mucins. The exopolysaccharide protects the microorganism from phagocytes and prevents antibiotic uptake. Consequently, it is a major pathogenic factor in these patients (3).

Alginates are degraded by a group of enzymes that catalyze the β-elimination of the 4-O-linked glycosidic bond, with formation of unsaturated uronic acid-containing oligosaccharides (6). Several of the bacteria that synthesize alginate-like polysaccharides also produce alginate lyases, but they cannot use the polymers as the sole carbon and energy source. Typically, alginate lyases have an absolute specificity for either D-mannuronic or L-guluronic acid at the nonreducing side of the bond to be cleaved but no limitation on the uronic acids at the reducing side (5). Although alginate lyase may prove useful in the elucidation of alginate molecular structure, its degrading action on alginate represents a drawback in the bioproduction of the polymer by fermentation. In studies of *P. aeruginosa*, it is necessary to look for inhibitors of alginate synthesis with potential use as therapeutic agents in cystic fibrosis patients. Therefore, research on this enzyme is important from different points of view.

In this paper, we describe the cloning of the *algL* gene encoding the alginate lyase from *A. chroococcum* and the purification and characterization of its product. We also report the conditions under which the enzyme can be overproduced in *Escherichia coli* and easily purified to electrophoretic homogeneity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *A. chroococcum* ATCC 4412 was grown at 30°C on nitrogen-free Burk's medium supplemented with 0.5% (wt/vol) sucrose as the sole energy and carbon source.

E. coli DH5α (Bethesda Research Laboratories) was used for all plasmid constructions, *E. coli* MC1061 (30) was used for gene library construction, and *E. coli* BL-21 (DE3) (40) was used as the host for pAPET-2 to produce alginate lyase. These strains were grown on Luria-Bertani (LB) and M9 minimal media containing ampicillin (100 μg/ml) as described by Sambrook et al. (37).

P. aeruginosa 8830, a gift from A. M. Chakrabarty (University of Illinois, Chicago), was grown as described by May and Chakrabarty (29) to isolate alginic acid, which was used as substrate for *A. chroococcum* alginate lyase.

Plasmid pRL500 (12) was used to construct the partial *A. chroococcum* gene library, and pBluescript II SK(+) (Stratagene) was used as cloning vector. Plasmid pET-3a, used for the expression of recombinant proteins, was from Novagen. pCL8, containing alginate lyase gene (*algL*) from *P. aeruginosa* (4), was a gift from A. M. Chakrabarty. Plasmids pAPL4.7 and pAPET-2, containing the *algL* gene from *A. chroococcum*, were constructed for this work.

DNA manipulation and Southern blot hybridization. Total DNA from *A. chroococcum* was isolated as described by Ausubel et al. (1). All DNA manipulations and *E. coli* transformations were performed by standard procedures (37). DNA fragments were purified from agarose gels by using the GeneClean kit (Bio 101, Inc.). For Southern hybridization, DNA was digested and fragments were electrophoresed on 0.7% agarose gels with the Tris-borate-EDTA buffer system (37). DNA was transferred to Z-probe membranes (Bio-Rad) under vacuum, and Southern blot hybridizations were performed as described by Ausubel et al.

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(1). DNA probes were ^{32}P labelled with a DNA-labelling kit (-dCTP) (Pharmacia) and [α-^{32}P]dCTP (Amersham).

Cloning of the *algL* gene. Genomic DNA from *A. chroococuum* was completely digested with *Eco*RI and fractionated on a 0.7% agarose gel. DNA fragments of approximately 2.0 to 4.0 kb were purified and ligated into the *Eco*RI site of pRL500. *E. coli* MC1061 was transformed with the ligation mixture, and transformants were grown on LB agar plates containing ampicillin. Transformants were screened for the presence of the *algL* gene by colony hybridization blotting with a 1.8-kb *Xba*I-*Eco*RI fragment from plasmid pCL8 as a probe.

DNA sequencing and nucleotide sequence analysis. DNA fragments containing the *algL* gene were subcloned into pBluescript II SK(+) and sequenced by the dideoxy chain termination method (38) with M13 universal and reverse oligonucleotides or synthetic oligonucleotides as primers. Sequencing reactions were carried out with Sequenase version 2.0 (US Biochemical Corp.). Nested unidirectional deletions were generated with the double-stranded nested-deletion kit (Pharmacia LKB). Both strands of DNA were sequenced.

Computer sequence analysis was carried out with the Genetics Computer Group software package (10). Amino acid sequences were compared with the FASTA program, and alignments were produced with the PileUp program and default parameters (33).

Expression of AlgL and purification of alginate lyase. To express AlgL protein, a PCR with plasmid template pAPL4.7 and primers designed to introduce the *Nde*I (5′-TATTCATATGAAGACCAGACTTGCCC-3′) and *Bam*HI (5′-CTTCGGGATCCCTGCGGAATACCAG-3′) restriction sites encompassing *algL* was performed by using a final volume of 50 μl that contained 1.5 ng of DNA from plasmid pAPL4.7, 0.2 mM each deoxynucleoside triphosphate, 50 pmol of each oligonucleotide, and 2.5 U of *Taq* polymerase and 1× buffer (Boehringer).

An expected PCR product of 0.2 kb containing *Bam*HI and *Nde*I sites was amplified. It was digested with *Nde*I and *Bam*HI, ethanol precipitated, and ligated into a similarly digested and phosphatase-treated pET-3a expression plasmid. Possible constructs were confirmed by restriction digestion and sequencing.

To create pAPET-2, a 1.8-kb *Bst*XI-*Eco*RV fragment from pAPL4.7 (containing the *algL* gene) was isolated and cloned in pET-3a (containing the PCR product) previously digested with *Bam*HI, refilled by a Klenow reaction, and finally digested with *Bst*XI. This plasmid contained the whole *A. chroococcum algL* gene under the control of the T7/*lac* promoter; in consequence, its expression was inducible by isopropyl-β-D-thiogalactopyranoside (IPTG).

E. coli BL-21 (DE3) cells were transformed with plasmid pAPET-2. Transformation mixtures were used to directly inoculate 2 liters of M9 minimal medium containing 100 μg of ampicillin per ml. The culture was incubated at 30°C to an absorbance at 550 nm (A_{550}) of 0.5; at this time, T7 polymerase was induced by adding IPTG at a final concentration of 1 mM, and cells were incubated for another 3 h at 30°C. Then cells were collected by centrifugation (8,000 × *g* for 5 min), and the periplasmic fraction was prepared as described by Efterkhar and Schiller (11). The periplasmic proteins were loaded onto a hydroxyapatite column (1.8 by 14 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.5). The adsorbed alginate lyase activity was eluted with a linear gradient of 0.1 to 1.0 M potassium phosphate buffer (pH 7.5). Most active fractions were pooled and loaded onto a phenyl-Sepharose column (1 by 8 cm) equilibrated with 25 mM Tris-HCl buffer (pH 8.2) containing 1 M NaCl. The alginate lyase activity was eluted within the void volume of this column. Most active fractions were combined and used as the purified AlgL enzyme.

Alginate lyase assay. As the substrate for the enzyme assay, alginic acid from either *Macrocystis pyrifera* (60% mannuronate; Sigma), *A. chroococcum*, or *P. aeruginosa* was used. Alginic acid from *A. chroococcum* was prepared as described by Jarman et al. (22), and that from *P. aeruginosa* was prepared as described by May and Chakrabarty (29). When needed, the substrate from *P. aeruginosa* was deacetylated before being used as substrate (16). Alginate lyase activity was quantitatively measured by the thiobarbituric acid method (42). Enzyme preparations (5 to 15 μg of total protein) in 50 mM Tris-HCl buffer (pH 7.5)–0.2 M $MgCl_2$ containing substrate (0.1 mg) were incubated at 30°C for 15 min. One unit of enzyme activity is defined as the amount of enzyme required to generate 1 μmol of β-formylpyruvate per min. The protein concentration was measured by the method of Bradford (8) with bovine serum albumin (Sigma) as the standard.

Alginate lyase plate assay. Alginate lyase-producing *E. coli* strains were identified by being grown at 37°C on LB plates containing 1% agarose, alginate from the seaweed *M. pyrifera* (2 mg/ml), and 100 μg of ampicillin per ml. The plates were stained by flooding with 10% (wt/vol) cetylpyridinium chloride, and clear zones of depolymerization on a white background were observed, indicating lyase activity (17).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was done by the method of Laemmli (24). Enzymatic activity after SDS-PAGE and subsequent renaturation were visualized exactly as described previously (34). All PAGE runs were performed at room temperature with a Bio-Rad mini Protean slab gel apparatus.

Nucleotide sequence accession number. The nucleotide sequence of the *A. chroococcum algL* gene (see Fig. 3) has been deposited in the DDBJ, EMBL, and GenBank DNA databases under accession no. AJ223605.

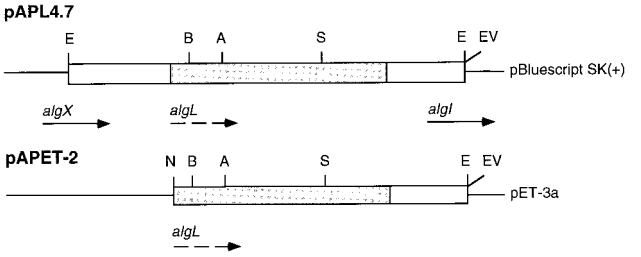

FIG. 1. Restriction map of pAPL4.7 and pAPET-2 containing the *A. chroococcum algL* gene. Thin lines and open bars correspond to vector plasmid DNA and cloned DNA fragments, respectively. The coding region is represented by solid bars. A broken arrow indicates the direction of the transcription of *algL*. E, *Eco*RI; A, *Apa*I; B, *Bst*XI, S, *Sac*I; N, *Nde*I; EV, *Eco*RV. Potential *algX* and *algI* genes upstream and downstream of *algL* gene are indicated by solid arrow underlines.

RESULTS AND DISCUSSION

Cloning of the *A. chroococcum algL* gene. To clone *algL*, total genomic DNA from *A. chroococcum* was digested and subjected to Southern blotting with, as the probe, a 1.8-kb *Xba*I-*Eco*RI fragment from plasmid pCL8, containing the *algL* gene from *P. aeruginosa* (4). A 3.2-kb *Eco*RI fragment hybridized with this probe (data not shown). Total *A. chroococcum* DNA was then digested with *Eco*RI, and fragments of approximately 2.0 to 4.0 kb were purified and ligated to pRL500 to construct a partial *A. chroococcum* gene library. Transformants in *E. coli* MC1061 were screened for the presence of the *algL* gene by using the same probe (see Materials and Methods). A positive clone was analyzed. This clone contained a 3.1-kb *Eco*RI insert, which was cloned into pBluescript to generate plasmid pAPL4.7 for further analysis (Fig. 1).

To determine whether this DNA fragment encoded the alginate lyase enzyme, *E. coli* DH5α cells were transformed with pAPL4.7 and tested for alginate lyase activity on LB plates containing alginate. A clear zone of depolymerization was observed around cells containing pAPL4.7. Untransformed *E. coli* DH5α did not synthesize an endogenous activity that was able to degrade alginate, and DH5α cells containing the pBluescript vector showed no clear zones (Fig. 2). Therefore, the 3.1-kb *Eco*RI fragment harbors the alginate lyase enzyme from *A. chroococcum*.

Nucleotide sequence of the *algL* gene. To determine if the 3.1-kb fragment contains the bonafide *algL* gene from *A. chroococcum*, nested deletions were generated from pAPL4.7 with exonuclease III and a number of the resulting clones were checked for alginate lyase activity to locate the *algL* gene in the

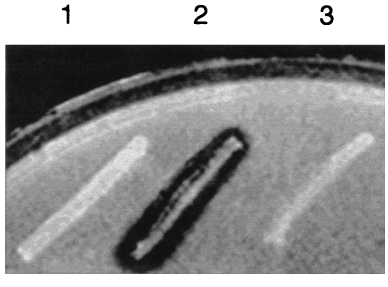

FIG. 2. Plate assay for alginate lyase activity. Cells were restreaked on LB plates containing alginate and assayed for alginate lyase activity (see Materials and Methods). 1, untransformed *E. coli* DH5α cells; 2, *E. coli* DH5α cells containing pAPL4.7 plasmid; 3, *E. coli* cells containing pBluescript SK(+) plasmid.

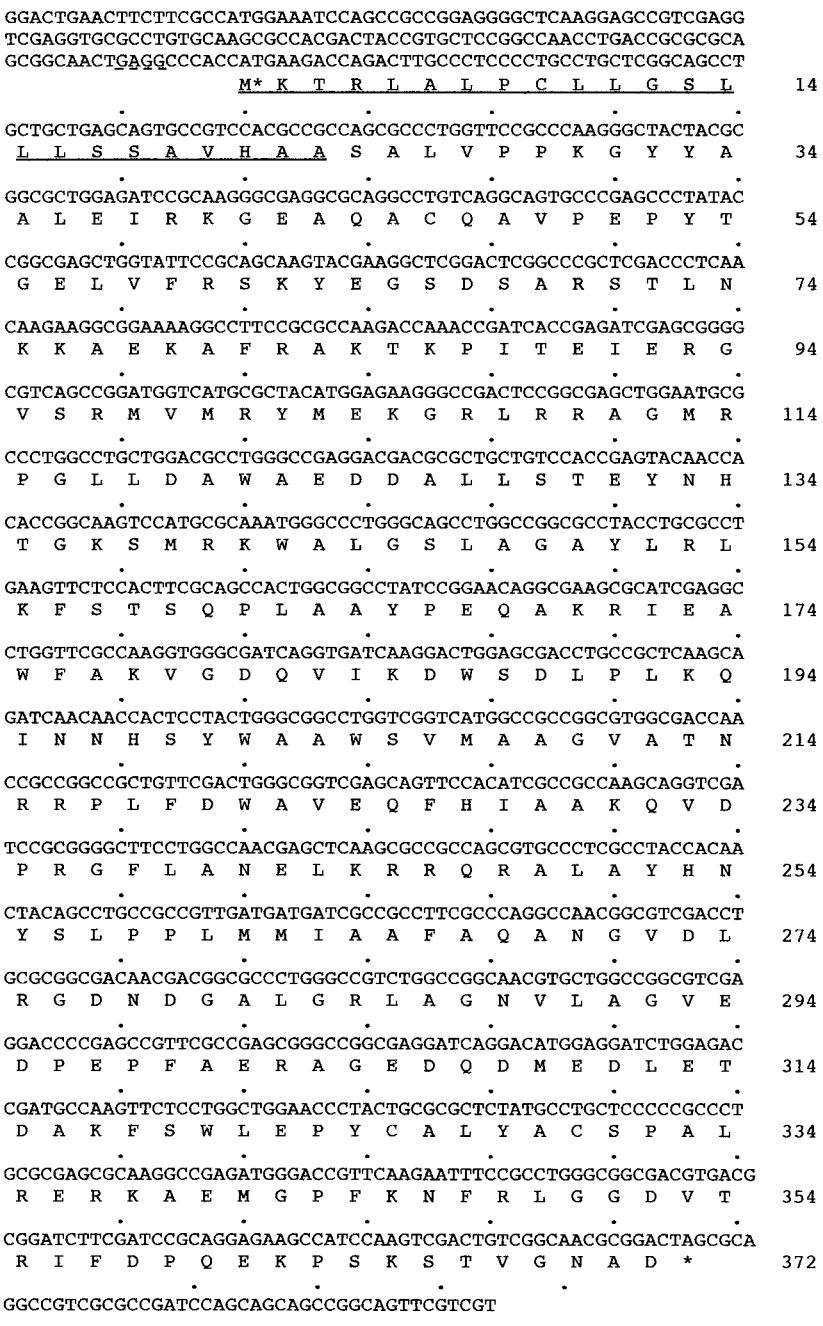

FIG. 3. Nucleotide sequence of *A. chroococcum algL* and deduced amino acid sequence of its product. The deduced amino acid sequence of AlgL is shown in single-letter code below the nucleotide sequence of *algL*. The possible Shine-Dalgarno sequence is indicated by a dotted underline. The initial methionine is indicated by an asterisk, and the possible signal peptide is indicated by a solid underline.

pAPL4.7 plasmid and were used for sequencing. The complete nucleotide sequence of *algL* gene and its translation of the open reading frame into a 372-residue amino acid sequence are shown in Fig. 3. The coding region ends with a TAG stop codon and it encodes a polypeptide with a calculated molecular mass of 42.98 kDa. As reported previously, the molecular mass of the alginate lyase from *A. chroococcum*, determined by activity staining after SDS-PAGE and subsequent renaturation, is 43 kDa (34) and the molecular mass estimated by gel filtration is about 46 kDa (35). A potential Shine-Dalgarno (GAGG) sequence lies just 7 nucleotides upstream from the start site. In the region of DNA upstream of the ATG codon, sequences related to the −35 and −10 consensus promoter regions were not identified. The G+C content of the nucleotide sequence is 67.2%, in good agreement with the value found for the *Azotobacter* genes, which is within 65 to 68% (2).

When sequences contained in plasmid pAPL4.7 flanking the *algL* open reading frame were sequenced and compared with other nucleotide sequences deposited in the data banks, it was observed that these sequences showed homologies to those of *P. aeruginosa algX* and *algI* genes, which are constituents of the alginate biosynthesis operon (14, 31). These data suggest that

TABLE 1. Purification of recombinant alginate lyase from *A. chroococcum* ATCC 4412[a]

Step	Total amt of protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
Periplasmic fraction	69.35	641.7	9.25	100	1
Hydroxyapatite	5.6	331.8	59.25	51.7	6.4
Phenyl-Sepharose	0.36	33.8	93.88	5.26	10.14

[a] The enzyme was purified from 5 g (fresh mass) of *E. coli* BL-21 (DE3) containing plasmid pAPET-2.

the *A. chroococcum algL* gene is probably located in an operon and has, as flanking genes, the *algX* gene at its 5′ end and the *algI* gene at its 3′ end. The existence of an alginate biosynthesis operon has also been described for *A. vinelandii* (13). The sequence of the *algXLIVFA* operon from *A. vinelandii* (as appeared in the GenBank database) indicates that the alginate biosynthesis operon in this organism has the same structure as the one described in the present report for *A. chroococcum*. The flanking sequences of the *A. chroococcum algL* gene show a high homology to the recently reported sequence of the *algXLIVFA* operon from *A. vinelandii*.

Deduced amino acid sequence of AlgL. The deduced protein sequence of AlgL was compared with entries in the GenBank and SWISS-PROT databases. Multiple alignment of several alginate lyase sequences indicates that *A. chroococcum* AlgL is more similar to *P. aeruginosa* AlgL (63.6% identity) than to *K. pneumoniae* AlgL (16.3% identity), *P. alginovora* ΔlgL (16.3% identity), or *Photobacterium* ΔlgL (18.8%). As mentioned above, the *A. vinelandii* AlgL deduced amino acid sequence (GenBank) is very similar (90% identity) to that from *A. chroococcum*.

The presence at the N-terminal region of a signal peptide of 20 to 30 amino acids, rich in hydrophobic residues, had been described for several alginate lyases (4, 27, 28, 39). This signal peptide has not been experimentally investigated in *A. chroococcum*, but the hydrophobicity profile deduced from the *algL* gene shows a highly hydrophobic region of 25 amino acids at the amino-terminal end. A potential cleavage site appears between amino acid residues Ala^{22} and Ala^{23}. This zone has high homology to the amino acid sequence of the signal peptide described for *P. aeruginosa*. These data suggest the existence of a signal peptide in *A. chroococcum* alginate lyase and the maturing of this protein. The mature protein would then begin at Ala^{23}. Processing and export through the inner membrane are consistent with the periplasmic location of AlgL in *A. chroococcum* (35) and its purification from the periplasmic fraction from *E. coli*.

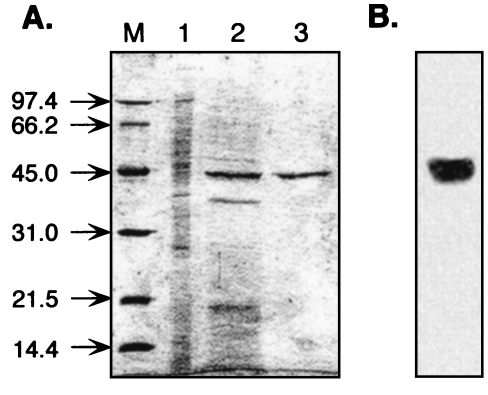

FIG. 4. SDS-PAGE analysis of the recombinant *A. chroococcum* AlgL protein. (A) Gel stained with Coomassie brilliant blue R-250; (B) alginate lyase activity detected on a gel containing 1% alginic acid. Lanes: M, molecular mass markers; 1, periplasmic fraction (10 μg); 2, hydroxyapatite column AlgL fraction (10 μg); 3, phenyl-Sepharose AlgL fraction (10 μg).

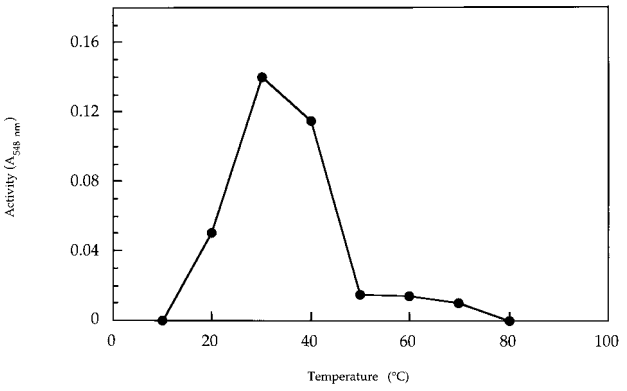

FIG. 5. Optimum temperature for the recombinant alginate lyase assay. Aliquots (25 μl) of an enzyme preparation (1.5 mU/μl) were analyzed for activity at the indicated temperatures, and the values found after a 30-min assay were spectrographically recorded at 548 nm by the thiobarbituric acid method.

Purification and characterization of recombinant alginate lyase. The *algL* gene was expressed from the bacteriophage T7 polymerase promoter of pET-3a expression vector. The final construct, called pAPET-2, was transformed in *E. coli* BL21 (DE3), and the AlgL enzyme was produced following induction of T7 polymerase. The best conditions for the overexpression of the enzyme were direct culture inoculation from the transformation mixture, 30°C, initiation of induction by adding 1 mM IPTG at an A_{550} of 0.5, and an induction time of 3 h. These conditions prevented the overexpressed protein from aggregation into inclusion bodies.

The gene product of *algL* was purified 10.4-fold from the periplasm fraction of *E. coli* BL21 (DE3)(pAPET-2), with a recovery of 6% by hydroxyapatite chromatography and phenyl-Sepharose column chromatography (Table 1). The final preparation gave a single band on SDS-PAGE with a molecular mass of approximately 43 kDa (Fig. 4). This value is in good agreement with the value estimated from the deduced amino acid sequence of AlgL and with the value determined by activity staining in SDS-PAGE for the native AlgL from *A. chroococcum* (34).

In agreement with other reports, which have described the partial purification of AlgL from both *A. chroococcum* and *A. vinelandii* (23), attempts to purify the native protein to homogeneity were unsuccessful even though a variety of purification steps were used. In this case, the recovery of partially purified enzyme (0.06 mg/liter of culture) was also lower than the obtained with the overexpressed alginate lyase (0.36 mg/liter).

To characterize the purified enzyme, it was found that the optimum temperature for the recombinant enzyme assay is 30°C at pH 7.5 and that alginate lyase was inactive when analyzed at 70°C (Fig. 5). These values are rather similar to those for the native enzyme (data not shown).

The enzyme activity was measured in the presence of cations at 100 mM. Like the native protein (35), the activity of recombinant AlgL increased in the presence of Na^+ and K^+; the combined action of these cations and Mg^{2+} increased the activity 100-fold with respect to the control without added cations (Table 2). When these monovalent and divalent cations were used at concentrations in the range from 1 to 50 mM, the

TABLE 2. Effect of different monovalent and divalent cations on alginate lyase activity assay[a]

Cation added	Alginate lyase activity (A_{548})
None	0.010
K^+	0.173
Na^+	0.014
Mg^{2+}	0.017
K^+ plus Mg^{2+}	0.927
Mg^{2+} plus Na^+	0.018
K^+ plus Mg^{2+} plus Na^+	1.059

[a] Aliquots of a purified *A. chroococcum* alginate lyase preparation (30 mU) were assayed for enzyme activity in the presence of the indicated cations at 100 mM (final concentration) each. Other experimental conditions were as in the thiobarbituric acid assay, except that 10 mM Tris-HCl buffer (pH 7.5) was used.

increase in alginate lyase activity was not significant. Other cations, like Ca^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} or the presence of EDTA does not affect the enzymatic activity. This enhancing effect of Mg^{2+} on alginate lyase activity has been reported for the enzyme from *Klebsiella aerogenes* (25), *P. alginovora* (7), and a marine bacterium that has not been identified (36). In all these cases, the presence of Mg^{2+} is stimulative but is not essential for the activity. There are other situations, however, where the cations are strictly required. Thus, *Bacillus circulans* JBH2 alginate lyase requires Mg^{2+} (18), and a *Pseudomonas* sp., *P. aeruginosa*, and *Littorina* sp. alginate lyase depends on Ca^{2+} (16). The enzyme from *A. chroococcum* 4A1M is activated by Ca^{2+} and inhibited strongly by Hg^{2+} (19). It should be mentioned that the enzyme from *A. chroococcum* 4A1M has a molecular mass of 23 kDa by SDS-PAGE and 24 kDa by gel filtration. Therefore, its molecular mass is roughly half that of the enzyme found in, and isolated by us from, *A. chroococcum* ATCC 4412. Furthermore, the enzyme from strain 4A1M showed maximum activity at 60°C whereas the one used in the present work has maximum activity at 30°C.

The K_m value for the recombinant *A. chroococcum* alginate lyase was found to be 0.08 mM for uronic acids, which is in the range from 0.1 to 0.5 mM reported for other alginate lyases (16). From a Lineweaver-Burk double-reciprocal plot, a V_{max} value of 0.183 mM min^{-1} was calculated for the enzyme.

To study the substrate specificity, the recombinant alginate lyase was used to degrade a series of alginates and the activity was assayed. AlgL was 17-fold more active against alginate from the seaweed *M. pyriferia* than against that from *P. aeruginosa*. The enzymatic activity against the *A. chroococcum* alginate was lower than with both above-mentioned substrates. Alginate from *P. aeruginosa* was 37% acetylated, but that from *M. pyriferia* was completely deacetylated. Therefore, it is likely that the acetylation of the bacterial alginate confers some protection against depolymerization by alginase. In favor of this conclusion is the fact that after chemical deacetylation of the *Pseudomonas* alginate, *A. chroococcum* AlgL increased its activity around 10-fold.

Finally, the possible roles of alginate lyases in some environments and several potential applications for both the oligosaccharides derived from the action of alginase and the enzyme itself have recently been discussed (41). Along these lines, Murata et al. (32) reported that they obtained an alginate lyase in large quantity and suggested its possible value as a therapeutic agent for the treatment of cystic fibrosis patients infected with mucoid *P. aeruginosa*. In fact, the use of alginate lyases to degrade the alginate in the lungs of cystic fibrosis patients, allowing a major diffusion of antibiotic, has been recently reported (20). The alginate lyase from *A. chroococcum* could be used as therapeutic agent because, as mentioned above, it is able to degrade the alginate produced by *P. aeruginosa*.

ACKNOWLEDGMENTS

This work was supported by financial help from Plan Andaluz de Investigación and Consejo Superior de Investigaciones Científicas (Spain). A. Pascual was supported by a fellowship from the Spanish Ministry of Education.

We thank F. J. Cejudo and J. de la Cruz for critical reading of the manuscript.

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