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## Sequence of a gene encoding a (poly ManA) alginate lyase active on *Pseudomonas aeruginosa* alginate

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**Abstract:** The recombinant plasmid pAL-A3 bears a (poly ManA) alginate lyase-encoding gene that originates from the marine bacterium ATCC 433367 (Brown et al., Appl. Environ. Microbiol. (1991) 57, 1870–1872). The alginate lyase produced by *Escherichia coli* TC4 harbouring pAL-A3 was purified to protein homogeneity and the corresponding gene sequenced, giving access to the first known primary structure of an alginate lyase. The 265-amino acid residue alginate lyase showed lytic activity on a *Pseudomonas aeruginosa* alginate isolated from a cystic fibrosis patient. Unexpectedly, the alginate lyase thus characterized differed from that isolated from the culture medium of the bacterium ATCC 433367 (Romeo and Preston, Biochemistry (1986) 25, 8385–8391).

**Key words:** (poly ManA) Alginate lyase; Gene cloning; Primary structure; *Pseudomonas aeruginosa*

### Introduction

Alginate is a linear uronic acid polymer in which  $\beta$ -D-mannuronate and  $\alpha$ -L-guluronate are 1–4-linked to form blocks of polymannuronate (poly ManA), polyguluronate (poly GuLA) and random sequences [1,2]. Alginate forms up to 30% of the dry weight of brown seaweed. It is also synthesized as an exopolysaccharide by bac-

teria [1,2]. Bacterial alginates are mainly of the poly ManA type with *O*-acetyl groups on the 2 and/or 3 positions of D-mannuronate [3]. Alginate facilitates attachment of mucoid *Pseudomonas aeruginosa* strains to tracheal epithelium and respiratory mucins, protects the bacteria from phagocytes and prevents antibiotic uptake [4–6]. It is an important virulence factor in cystic fibrosis [7,8]. Use of an alginate-degrading lyase may have salutary effects on the clearance of the bacteria by antibiotic- and polymorphonuclear leukocyte-mediated killing [9–11]. Alginate lyases have been isolated from various sources [2]. They catalyse cleavage of the polysaccharide chain by a

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$\beta$ -elimination reaction which causes formation of a double bond between C<sub>4</sub> and C<sub>5</sub>, elimination of the 4-*O*-glycosidic bond and production of 4-deoxy-L-erythro-hex-4-ene pyranosyluronate at the non-reducing end of the resulting oligosaccharide [2]. Genes encoding alginate lyases have been cloned [12–15] but, at this time, a sequence has not been published.

As reported by Romeo and Preston [16], the extracellular (poly ManA) alginate lyase produced by the marine bacterium ATCC 433367 (an epiphyte of *Sargassum fluitans*) was purified to protein homogeneity. Subsequently, as reported by Brown et al. [12], an alginate lyase-encoding gene originating from the same bacterium was cloned in the form of a 4-kb insert in plasmid pAL-A3 and an alginate lyase was produced by *Escherichia coli* TC4 harbouring pAL-A3. As described in this paper, the alginate lyase expressed in the periplasm of *E. coli* TC4/pAL-A3 has been purified to protein homogeneity and the encoding gene has been sequenced, giving access to the primary structure of the protein. Unexpectedly, the sequenced gene encodes an alginate lyase which differs from that isolated from the culture medium of bacterium ATCC 433367.

## Materials and Methods

### Bacterial strain and growth conditions

*E. coli* TC4/pAL-A3 [12] was a gift from Dr. B.J. Brown, Medical Center, Department of Biological Chemistry, Ann Arbor, MI. The strain was grown at 37°C for 15 h in Luria-Bertani medium containing 50  $\mu$ g ampicillin per ml.

### Enzyme assays

*E. coli* colonies producing the alginate lyase were characterized by a sharply demarcated settling of the 'black medium' on plates containing 0.5% agar, 0.5% yeast extract (Difco), 1% high viscosity alginate of *Macrocystis pyrifera* (Sigma) and 0.5% animal charcoal (Serva) [17]. Solutions (0.4 ml) of alginate lyase in 0.1 M Tris · HCl (pH 7.5) containing 0.3% low-viscosity *M. pyrifera* alginate (Sigma) were incubated for 10 min at 37°C. The amounts of unsaturated non-reducing ends

produced by  $\beta$ -elimination (see Introduction) were measured as described [18]. After periodate oxidation (formation of  $\beta$ -formyl pyruvic acid) and reaction with thiobarbituric acid (Sigma), the chromophore was estimated spectrophotometrically at 548 nm. One enzyme unit produced 1  $\mu$ mol of non-reducing, unsaturated termini per min using 3-deoxy-D-manno-octulosonic acid (KDO, Sigma) as the standard. Protein was estimated [19] using bovine serum albumin as the standard.

### Enzyme purification

Cells from 1.5 l of a stationary phase culture were suspended in 120 ml of 30 mM Tris · HCl (pH 8.0) containing 20% sucrose, 5 mM EDTA and 16 mg lysozyme. The suspension was maintained at 0°C for 20 min, supplemented with 10 mM CaCl<sub>2</sub> and 0.4 M NaCl. Centrifugation yielded the periplasmic fraction which contained the alginate lyase (18.4 enzyme units; 155 mg total protein; specific activity 0.12 units per mg protein). The enzyme was purified through a two-step procedure. *Step 1.* The periplasmic fraction, dialysed against 10 mM Na phosphate, pH 7.0 (buffer T)/50 mM NaCl, was loaded on a 2.5 × 8-cm Bio-Rad hydroxyapatite column equilibrated against buffer T/50 mM NaCl. Using a linear gradient (6 bed volumes) from 100 to 450 mM Na phosphate, pH 7.0/50 mM NaCl (flow rate 80 ml h<sup>-1</sup>), the enzyme eluted at 300 mM phosphate. *Step 2.* The active fractions (pooled, concentrated and dialysed against buffer T) were loaded on a MonoQ HR 5/5 column equilibrated against buffer T (FPLC system, Pharmacia). Using a two-step NaCl gradient from 0 to 0.3 M NaCl in buffer T (20 bed volumes) and from 0.3 to 1 M NaCl in buffer T (16 bed volumes) (flow rate 1 ml min<sup>-1</sup>), the enzyme eluted at 0.15 M NaCl as a sharp, symmetrical peak. All the operations were carried out at 4°C except the FPLC chromatography which was performed at 20°C. The fractions were concentrated by ultrafiltration on Amicon YM-10 membrane.

### SDS-PAGE, amino acid sequencing and experimental pI

SDS-PAGE (15%) was carried out according to Laemmli and Favre [20]. Gels were stained

with PAGE blue 83. Others were electroblotted on Immobilon Millipore membranes using a Pharmacia LKB Novablot apparatus as described [21]. Membrane strips with the electrotransferred polypeptides were inserted in the sequencing chamber of an Applied Biosystems 470A amino acid microsequencer. The *pI* of the protein was measured by agarose gel slab electrofocusing using Pharmalyte 3–10 and the conditions recommended by Pharmacia.

#### Gene cloning and sequencing

pAL-A3 was the source of alginate lyase-encoding gene. Subcloning was done in pUCBM20 (Boehringer, Mannheim, FRG). *E. coli* RR1 [22] served as a host. Nucleotide sequencing was performed according to Sanger et al. [23] using M13 universal, reverse oligonucleotide or synthetic oligonucleotides as primers. Denaturation of double-stranded DNA was performed according to Zhang et al. [24]. Sequencing reactions were carried out by using the T7 sequencing kit (Pharmacia, LKB, Biotechnology, Uppsala, Sweden) with [<sup>35</sup>S]dATP labelling or the Autoread sequencing kit (Pharmacia, LKB Biotechnology) with fluorescent primers or incorporation of fluorescent dUTP. In this latter case, electrophoresis

was done with an A.L.F. apparatus (EMBL, Heidelberg, FRG) [25].

#### Homology searches and theoretical *pI*

Search through the protein sequence databases (PIR version 32) was made according to Pearson and Lipman [26] using the FASTA and TFASTA software packages. Theoretical *pI* was computed according to Devereux et al. [27] (GCG package).

#### Nucleotide sequence accession number

The EMBL accession number for the nucleotide sequence shown in Fig. 3 is X70036.

## Results

#### Purification and properties of the alginate lyase

*E. coli* TC4/pAL-A3 produced alginate lyase in the periplasm at a maximal level of 0.012 units per ml of culture (specific activity 0.12 units per mg protein) during the stationary phase. The enzyme was purified as described in Materials and Methods. The yields were 83% and 17% and the purification factors were 43 and 328 after step 1 (Fig. 1A) and step 2 (Fig. 1B), respectively. The purified enzyme had a specific activity of 40 units

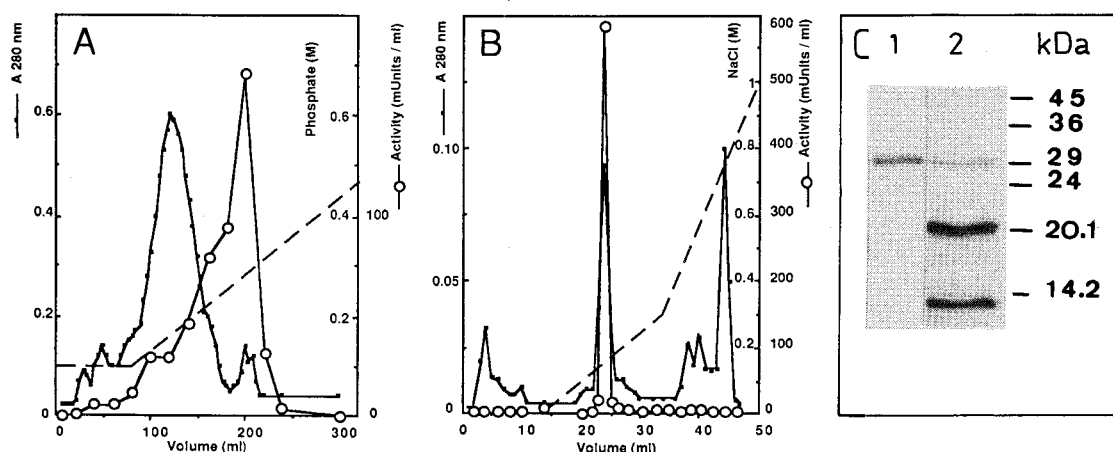


Fig. 1. Purification of the alginate lyase and SDS-PAGE of the purified enzyme in the absence and the presence of  $\beta$ -mercaptoethanol (A) and (B), Hydroxyapatite and MonoQ HR 5/5 chromatography, respectively. For details, see Materials and Methods. (C) SDS-PAGE (15%) of the MonoQ HR 5/5-purified enzyme previously boiled for 5 min in denaturing buffer without  $\beta$ -mercaptoethanol (lane 1) and in the presence of 5%  $\beta$ -mercaptoethanol (lane 2). The gels were stained with Coomassie brilliant blue.

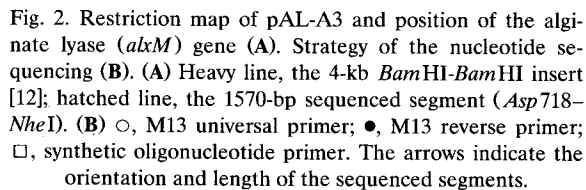


Fig. 3. Nucleotide sequence of the 1570-bp DNA fragment *Asp*718 (position 2088 in pAl-A3)–*Nhe*I (position 3658) and deduced amino acid sequence of the alginate lyase. The signal peptide is numbered negatively. The mature alginate lyase starts at G1.

it gave rise to two polypeptides of 20 kDa and 10 kDa, respectively (Fig. 1C, lane 2). The N-terminal region of the 20-kDa polypeptide fragment had the sequence GVEFSNP. The 10-kDa polypeptide had ragged KDKEMXXADV N-termini.

### Nucleotide sequence of the alginate-lyase-encoding gene and primary structure of the expressed protein

Positioning of the alginate-lyase-encoding gene in the 4-kDa insert of pAL-A3 (the restriction map of which is shown in Fig. 2A) was made by subcloning experiments. The 1600-bp *Asp*718 (position 408)–*Asp*718 (position 2088) fragment 1,

the 2700-bp *Cla*I (position 751)–*Cla*I (position 3473) fragment 2 and the 3100-bp *Sal*I (position 1221)–*Sal*I (position 4352) fragment 3 were ligated to pUCBM20 previously cut with *Asp*718, *Cla*I and *Sal*I, respectively. Similarly, the 1570-bp *Asp*718 (position 2088)–*Nhe*I (position 3658) fragment 4 was ligated to pUCBM20 previously cut with *Asp*718 and *Xba*I. *E. coli* recombinants were grown on 'black medium' plates. Those containing fragment 3 or 4 gave a positive alginate lyase test. Fragment 4 was sequenced using the strategy shown in Fig. 2B. The results are shown in Fig. 3. A 855-nucleotide residue ORF translated into a 285-amino acid residue protein precursor. The 20-amino acid residue N-terminal region had the function of a signal peptide. The sequences of the G1-P7 and K178-V187 polypeptide stretches of the mature protein were identical to those of the N-terminal regions of the 20-kDa and 10-kDa fragments, respectively. The protein had a high content in diamino acid residues (H, K and R; 14.7%). The computed theoretical *pI* value (8.16) was 2 units higher than the experimental *pI* value (approx. 6.0). A search of the protein sequence databases, revealed no significant similarity of the alginate lyase with known proteins. Based on the solvent accessibility profile (not shown), the decapeptide C169–C183 was well exposed at the surface of the protein.

## Discussion

The 4-kb DNA insert present in the recombinant plasmid pAL-A3, originated from the marine bacterium ATCC 433367 and bears an alginate lyase-encoding gene with its own promoter [12]. Accordingly, *E. coli* TC4/pAL-A3 produces an alginate lyase in its periplasm. Maximal level of enzyme production is achieved during the stationary phase. The purified 30-kDa enzyme exhibits high lytic activity on a *P. aeruginosa* alginate isolated from a cystic fibrosis patient [28]. Its N-terminal sequence is GVEFSNP.

As derived from subcloning experiments, the 1.6-kb DNA segment – which extends from position 2088 to position 3658 in pAL-A3 – contains

an alginate lyase-encoding gene. The ORF extends from position 2732 to position 3587. It encodes a 285-amino acid residue protein precursor which, upon cleavage of the signal peptide, gives rise to a 265-amino acid residue mature protein, the N-terminal region of which has the expected GVEFSNP sequence. Contrary to expectations, this alginate lyase differs from that isolated originally by Romeo and Preston [16] from the same ATCC 433367 bacterium. The two enzymes have varying N-terminal regions (GVEFSNP vs. DSAPYD), they behave differently on MonoQ HR 5/5 chromatography and they have distinct *pI* values (approx. 6.0 vs. approx. 4.5). The most likely explanation is that the bacterium ATCC 433367 produces more than one alginate lyase. The alginate lyase isolated from the culture filtrate of this organism and that isolated from the periplasm of *E. coli*/pAL-A3 might be distinct proteins encoded by distinct genes.

One peculiar feature of the alginate lyase produced by *E. coli* TC4/pAL-A3 is that proteolytic cleavage of the C169–C183 surface loop and chemical cleavage of the C169–C183 SS bridge result in the fragmentation of the 30-kDa protein into two polypeptides of 20 kDa and 10 kDa, respectively. The fact that the 10-kDa fragment has ragged N-termini is strong evidence of proteolytic cleavage of the loop. The existence of an SS bridge rests upon the effects of the reducing agent. Whether the enzyme is exported to the periplasm as an intact or a catalytically-active nicked polypeptide chain (with a cleaved and partially degraded C161–C183 surface loop) is unknown. The C-terminal region of the protein is probably essential for enzyme activity. Clones in which the alginate lyase gene is cleaved at *Cla*I (position 3473 in pAL-A3) and encodes a protein truncated downstream from I228, give a negative alginate lyase test when grown on 'black medium'.

The theoretical *pI* (8.16) value of the protein (computed on the basis of the amino acid sequence derived from gene sequencing) is about 2 units higher than the experimental *pI* (approx. 6.0) value. Elimination of the octapeptide K170–N177 of the C169–C183 loop would give rise to a nicked protein with a theoretical *pI* value of 7.0. Depending on the spatial arrangement of the

secondary structure elements, internal salt bridges may also contribute to a decreased *pI* value.

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