Cloning, sequence analysis and expression in \textit{Escherichia coli} of a gene encoding an alginate lyase from \textit{Pseudomonas} sp. OS-ALG-9

HIDEAKI MAKI, ATSUTOSHI MORI, KAZUHITO FUJIYAMA, SHINICHI KINOSHITA\textsuperscript{†} and TOSHIOMI YOSHIDA\textsuperscript{*}

International Center of Cooperative Research in Biotechnology, Japan, Faculty of Engineering, Osaka University, Suita, Osaka 565, Japan

(Received 9 December 1992; accepted 8 January 1993)

A gene (\textit{aly}) encoding alginate lyase (ALY; EC 4.2.2.3) was isolated from a library constructed with the cosmid vector pHCl79 and Sau3A1-digested genomic DNA of \textit{Pseudomonas} sp. OS-ALG-9. Successive subcloning of the \textit{aly}-containing cosmid enabled us to locate the gene on a 2.3 kb \textit{HpaI} fragment. Nucleotide sequencing of this fragment revealed a single open reading frame (ORF) of 1365 bp. The directly determined N-terminal amino acid sequence of the ALY protein purified from \textit{Pseudomonas} sp. OS-ALG-9 was found in the amino acid sequence deduced from this ORF between nucleotides 282 and 366. Expression of \textit{aly} was induced by IPTG in \textit{Escherichia coli} and leakage of the enzyme into the extracellular milieu was significantly enhanced by addition of glycine to the growth medium. The ALY enzyme had a greater specificity for the homopolymer of mannuronate than for that of guluronate.

Introduction

Alginate is an uronic acid co-polymer comprising (1-4)-linked \(\alpha\)-L-guluronate (G) and \(\beta\)-D-mannuronate (M) (Haug \& Larsen, 1962). These moieties can be arranged in either homopolymeric (polyG or polyM) or heteropolymeric structures consisting of random sequences of G and M (Haug \textit{et al.}, 1967). Because of its high viscosity and gelling properties with calcium ions, alginate is used widely in the food, pharmaceutical and medical industries. Alginate-depolymerizing enzymes have been isolated from fungi (Wainwright \& Sherbrook-Cox, 1981), marine molluscs (Nakada \& Sweeney, 1967; Nishizawa \textit{et al.}, 1968) and seaweeds (Madgwick \textit{et al.}, 1973; Shiraiwa \textit{et al.}, 1975). Most alginate lyase (ALY) enzymes, however, have been purified and characterized from bacteria (Davidson \textit{et al.}, 1976; Romeo \& Preston, 1986; Lange \textit{et al.}, 1989). These were found to be lyases instead of hydrolases, catalysing the \(\beta\)-elimination of 4-O-linked glycosidic bonds (Preiss \& Ashwell, 1962). Although most ALYs are endo-acting enzymes, some exo-acting enzymes have been reported (Doubet \& Quatrano, 1984).

A gene encoding a guluronate-specific ALY from \textit{Klebsiella pneumoniae} has been cloned and expressed in \textit{Escherichia coli} (Caswell \textit{et al.}, 1989). This was followed by the cloning and expression in \textit{E. coli} of the gene for a mannuronate-specific ALY from a marine bacterium (Brown \textit{et al.}, 1991). Neither of these studies reported the nucleotide sequences of the cloned genes, and therefore it has not been possible to compare the structural similarities of these enzymes. We have previously reported the isolation and characterization of an alginate-degrading bacterial strain, \textit{Pseudomonas} sp. OS-ALG-9, and described the culture conditions for ALY production. This strain produces both extra- and intracellular ALYs, and one of the intracellular enzymes was purified and characterized (Kinoshita \textit{et al.}, 1991). In this paper, we report the nucleotide sequence of a gene, designated \textit{aly}, for ALY from \textit{Pseudomonas} sp. OS-ALG-9. The data provide new insights into the structure–functions relationship of bacterial ALY.
Methods

Bacterial strains, vectors, media and culture conditions. The bacterial strains and vectors used in this study are summarized in Table 1. Culture conditions for the Pseudomonas sp. were described previously (Kinoshita et al., 1991). E. coli was grown in Luria-Bertani (LB) medium at 30 or 37 °C.

DNA manipulations. Plasmid DNA was prepared using the Brij lysis method (Clewell & Helinski, 1969), followed by CsCl/ethidium bromide ultracentrifugation. Mini-preparation of plasmid DNA was done using the method of Birnboim and Doly (1979). Routine tasks such as restriction endonuclease digestion, ligation and agarose gel electrophoresis were carried out according to Sambrook & al., 1989).

Construction of a genomic library of Pseudomonas sp. OS-ALG-9. Cells were grown at 30 °C in a 2 litre conical flask containing 500 ml of medium on a reciprocal shaker as described previously (Kinoshita et al., 1991). The cells were harvested by centrifugation after approximately 19 h cultivation and washed once with 25% (w/v) sucrose in 0.4 M-Tris/HCl, pH 8.0. Genomic DNA was extracted from the cells according to the method of Caswell et al. (1989). Purified genomic DNA was partially digested with Sau3AI and fractionated by CsCl/ethidium bromide ultracentrifugation. DNA fragment size in each fraction was examined by gel electrophoresis using a HindIII digest of λ DNA as molecular size standards. Fractions containing DNA fragments greater than 23 kb were pooled and ligated to the cosmid pHC79 (Hohn & Collins, 1980), which had been digested with BamHI and dephosphorylated with calf intestine alkaline phosphatase. The resulting concatamers were packaged in vivo into λ phage heads using a Gigapack kit (Promega). After transduction of E. coli LE392, colonies harbouring cosmids were selected on LB-agar plates containing ampicillin (50 μg ml⁻¹).

Assay for alginate lyase activity. Plate assays were done according to the method of Gaesens & Wusteman (1990). Each transductant was transferred onto an LB-agar plate with ampicillin (100 μg ml⁻¹) and incubated overnight at 37 °C. After growth was observed, 100 mg cetylpyridinium chloride ml⁻¹ was overlayed on the plates and allowed to stand at room temperature for approximately 30 min. Clear zones were observed around ALY-producing colonies. A solution of intracellular ALY was prepared as follows. E. coli or the Pseudomonas sp. were grown in 20 ml medium in 100 ml conical flasks at 30 °C. When the aly gene was inserted downstream of the lac promoter, ALY expression was induced by IPTG after 5 h cultivation, when required, followed by addition of glycine to 1% (w/v). The cells were harvested by centrifugation and disrupted by sonication. Cell debris was removed by centrifugation and the resulting clear lysate was used as a crude enzyme preparation for this study. In order to study the distribution of alginate lyase activity in E. coli JM109, aspartate aminotransferase (AspAT) was used as an intracellular marker protein. The enzyme activity of AspAT was determined by a malate-dehydrogenase-coupled method (Karmen, 1955). The enzyme assays were reproducible ±10%.

ALY activity was quantified by the thiobarbituric acid (TBA) method developed by Weissbach & Hurwitz (1959). The enzyme solution was incubated with 125 mg sodium alginate ml⁻¹ in 12.5 mM-Tris/HCl, pH 8.0, for 10 min at 30 °C. One unit of activity is defined as the amount of enzyme required to generate 1 μmol β-formylpyruvate by the periodate treatment in 1 min. ALY activity in the cell extract is shown in Fig. 1 as activity relative to the absorbance at 280 nm of crude cell extract [mUnits (μM⁻¹)]. The enzyme assays were reproducible ±25%.

Homopolymeric block regions of β-mannuronate and ε-guluronate were prepared using the method of Haug et al. (1967). The uronate compositions of each prepared block region were checked by 1H NMR spectroscopy (Grasdalen et al., 1979).

Subcloning and DNA sequencing. The two recombinant plasmids pAL20 and pAL12 (pUC18 derivatives containing the 2.3 kb HpaI fragment of aly) were used to prepare deletions for locating aly and for nucleotide sequencing. Unidirectional deletions of the 2.3 kb HpaI insert were created by BamHI-KpnI cleavage of the pUC18 portion of pAL20 and pAL12 followed by exonuclease III and mungbean exonuclease digestion. E. coli was transformed by the deletion mutants and screened by sodium alginate plate assay. For DNA sequencing, after exonuclease III and mungbean exonuclease digestion, the deleted fragments were size-selected by agarose gel electrophoresis and subcloned into M13mp19 vector. The DNA sequence was determined by the dideoxy chain termination method (Sanger et al., 1977) using the Sequenase sequencing kit (USB). The DNA sequence in the 5'-flanking region of the gene was also determined using an automated DNA sequencing system (model 373A, Applied Biosystems).

Purification of ALY from Pseudomonas sp. OS-ALG-9. Intracellular ALY was partially purified from cell extract of the Pseudomonas sp.
**Alginate lyase gene of Pseudomonas sp. OS-ALG-9**

**Results and Discussion**

**Cloning of Pseudomonas sp. OS-ALG-9 aly in E. coli**

Using the cosmid vector pHC79, a genomic library of *Pseudomonas* sp. OS-ALG-9 was constructed. The 3100 transductants were screened on sodium alginate plates and 10 ALY-positive colonies were obtained. The recombinant cosmid DNAs from these colonies were extracted and used to re-transform *E. coli*. Only one cosmid, pAL28, was able to stably transform the host to the ALY-positive phenotype. The overall size of the insert DNA in pAL28 was about 35 kb. To obtain a smaller insert, fragments of pAL28 digested to completion with *KpnI* were ligated to *KpnI*-digested pUC18. *E. coli* HB101 transformed with this recombinant plasmid was re-screened for enzyme activity using the sodium alginate plate assay. The plate assay yielded ALY-positive clones that contained a 8 kb *KpnI* fragment in pUC18. The recombinant plasmid was designated pAL5 and was used for further subcloning. Restriction enzyme mapping of pAL5 showed that *BglII* divided the 8 kb *KpnI* fragment into three fragments: a 3.8 kb *BglII*-*BglII* fragment, a 4 kb *BglII*-*KpnI* fragment and a *BglII*-*KpnI* fragment approximately 0.2 kb in size (Fig. 1a). *E. coli* cells transformed with pAL10 (pUC18 containing the 4 kb *BglII*-*BglII* fragment) were ALY-positive, and cells containing a 2.3 kb *HpaI* fragment of pAL10 subcloned into the *HincII* site of pUC18 were also ALY-positive. Plasmids bearing the 2.3 kb *HpaI* fragment in the same and reverse orientation with respect to the *lacZp* on pUC were designated pAL20 and pAL12, respectively. When *E. coli* JM109 carrying pAL12 and pAL20 respectively was not induced with IPTG, the activities of these two were the same (data not shown). However, the activity in bacteria harbouring pAL20 was induced 4-fold with IPTG, while the activity in pAL12-

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**Fig. 1.** (a) Restriction map of an 8 kb *KpnI* fragment in pAL5, and ALY activities in cell-free extracts of *E. coli* carrying pAL5, pAL10, pAL10' and pAL20. The ALY activity of a recombinant cosmid clone, *E. coli* HB101 carrying pAL28, was 0.36 milliUnits (A_{260}^{-1})

(b) Locating *aly* to a 2.3 kb *HpaI* fragment. The plasmids were prepared by exonuclease III and mungbean nuclease digestion, and introduced into *E. coli* JM109, followed by screening on sodium alginate plates. pAL205 and pAL203 were deleted from the 5' end of the 2.3 kb *HpaI* fragment encoding ALY. pAL122 and pAL123 were deleted from the 3' end of the fragment. Activity of each deletion mutant was detected by plate assay and is shown as + (strong), + (weak) and − (not detected).
containing cells was unaffected by IPTG (data not shown). This suggests that there is a promoter-like sequence in the pseudomonad insert in pAL20 and that *aly* might be expressed under the control of this sequence. On the other hand, *aly* in pAL20 is subject to *lacZp* control. As shown in Fig. 1 (b), deletion derivatives of the 2.3 kb *HpaI* insert of pAL20 and pAL20 were created. The resulting deletion derivatives, pAL20 (300 bp deletion) and pAL122 (600 bp deletion), both retained ALY activity, while the larger deletions carried in pAL20 and pAL12 (Fig. 1b). These results indicated that the region of *aly* essential for activity is located within approximately 1.4 kb (Fig. 1b). The cloned *HpaI* fragment of pAL20 was hybridized to a single 4 kb *BglII* restriction fragment from *Pseudomonas* sp. OS-ALG-9 (data not shown). This confirmed that the cloned fragment in pAL20 originated from the *Pseudomonas* sp. and demonstrates that no major rearrangements have occurred in the coding fragment. Compared to the original cosmid clone pAL28, subclones bearing smaller fragments of the insert (e.g. pAL5, pAL10 and pAL20) showed higher enzyme activities. This may be due to an increase in plasmid copy number or to the fact that the 5' flanking region are underlined in the 5' flanking region. The potential Shine-Dalgarno sequence is marked with dots below the DNA sequence.

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**Fig. 2.** Nucleotide sequence of the *aly* gene and deduced amino acid sequence of ALY enzyme from *Pseudomonas* sp. OS-ALG-9. The nucleotide sequence of both subclones is closer to the *Pseudomonas* sp. DNA insert was used to probe the plasmid DNA. Unique restriction sites *EcoT141* and *BstEII* (indicated by underline in the 5' flanking region) and deduced amino acid sequence of ALY enzyme from *Pseudomonas* sp. OS-ALG-9. The nucleotide sequence of both subclones are underlined. Lines with arrowheads indicate sequences capable of forming stem and loop structures. The determined amino acid sequence of purified protein is underlined below each residue. The potential Shine-Dalgarno sequence is marked with dots below the DNA sequence.
Alginate lyase gene of Pseudomonas sp. OS-ALG-9

Fig. 3. In vitro transcription/translation analysis of aly. Supercoiled template DNA was used to programme an E. coli 30S cell-free extract. The reaction mixtures and proteins partially purified from the Pseudomonas sp. were separated on the same SDS-polyacrylamide gel. An autoradiograph of [35S]methionine incorporation into proteins produced by the plasmids pAT153 (lane 1) and pAL20 (lane 2) is shown. pAT153 was supplied as a control from Amersham International. The ALY protein was stained with Coomassie Brilliant Blue (lane 3) and its position is marked. This protein was subjected to N-terminal sequence analysis and the determined sequence was identical with the sequence deduced from the DNA sequence. Molecular mass standards (lane 4) are Bio-Rad prestained markers.

The calculation of potential stem and loop structures resembling rho-dependent terminators were observed downstream of the ORF. A consensus promoter sequence has not been identified in Pseudomonas sp. (Deretic et al., 1987). Although no obvious promoter sequences (i.e. −10 or −35 regions) for σ70-type or any other known prokaryotic RNA polymerase (Helmann & Chamberlin, 1988) could be observed in the 5′-non-coding region of this sequence, potential −35 and −10 regions occurred at nucleotide 66–71 and 87–92. A possible Shine–Dalgarno sequence is located at 116–120. The putative amino acid sequence for ALY, consisting of 398 residues, is aligned below the nucleotide sequence (Fig. 2). The calculated molecular mass of the ALY protein, as deduced from the ORF, is 50620 Da. The N-terminal amino acid sequence of the enzyme purified from Pseudomonas sp. OS-ALG-9 cells was determined as N-H-Glu-Lys-X-Tyr-Thr-Ile-Ser-Ala-X-Glu-Leu-X-Gln-X-Asn-X-Met-Pro-X-X-Lys-Val-X-Met-Lys-Ser-Gly (unidentified residues are shown as X). This amino acid sequence is found in the ORF between nucleotides 282 and 366. The polypeptide starting at 41 (Glu residue) and ending at 455 (Lys) has a calculated molecular mass of 46361 Da. This size is consistent with that of the enzyme purified from Pseudomonas sp. OS-ALG-9 in this experiment (Fig. 3) as well as previously reported by Kinoshita et al. (1991). The primary translation product would be a larger precursor with an additional 40-residue N-terminal leader sequence. In vitro transcription/translation of pAL20 gave [35S]methionine-labelled bands at about 50 kDa (Fig. 3), slightly larger than the purified enzyme. The 50 kDa band must correspond to aly on the HpaI fragment of pAL20. The 50 kDa protein is thus likely to be a precursor form of ALY containing a signal peptide. The putative leader sequence is very similar in size and organization (e.g. a positively charged N-terminal region, hydrophobic region and a small residue, Ser, beside a processed Glu residue) to a signal sequence which is necessary for and removed during protein export of periplasmic proteins (von Heijne, 1986). A search of nucleotide and protein sequence databases revealed no significant homology to any known protein or DNA sequences.

Expression of aly in E. coli.

Expression of aly in E. coli harbouring pAL205 was induced about 7-fold by addition of IPTG (data not shown). The enzyme activity of these induced E. coli cells was found in the extracellular (culture broth) and intracellular (cell-free extract) fractions, and each activity was 2- and 20-fold higher, respectively than that of the Pseudomonas sp. (Table 2). It is generally considered that E. coli does not secrete proteins into the extracellular milieu. However, Ikura (1986) has shown that leakage of

Table 2. Distribution of ALY produced by Pseudomonas sp. OS-ALG-9 and E. coli JM109(pAL205)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Extracellular [milliUnits (ml culture)⁻¹]</th>
<th>Intracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas sp. OS-ALG-9</td>
<td>5.9</td>
<td>5.2</td>
</tr>
<tr>
<td>Escherichia coli JM109(pAL205)*</td>
<td>10.5</td>
<td>11.7</td>
</tr>
</tbody>
</table>

* E. coli JM109(pAL205) was grown at 30 °C in the presence of 1 mM-IPTG.
an intracellular enzyme can be enhanced by addition of glycine to the growth medium. As shown in Table 3, upon addition of glycine the total ALY activity increased 2-fold and the activity in the extracellular fraction increased sharply up to approximately 40% of the total activity. While aspartate aminotransferase (AspAT), an intracellular enzyme, was also found in the extracellular fraction upon addition of glycine, less AspAT than ALY leaked out. In the case of the ALY cloned from *Klebsiella pneumoniae*, more than 90% of the enzyme expressed in *E. coli* was leaked out of the cell, even without addition of glycine (Caswell et al., 1989).

### Substrate specificity of ALY produced in *E. coli*

Of the plasmid constructs shown in Fig. 2, *E. coli* JM109 carrying pAL205 expressed the highest level of ALY activity. ALY, expressed in *E. coli* JM109 carrying pAL205 and secreted into the culture broth after addition of glycine, was used to determine the substrate specificity of the enzyme, using prepared D-mannuronate-rich and L-guluronate-rich homopolymer as substrates. The homogeneity of uronic acid composition in each prepared homopolymeric glycuronan was confirmed by $^1$H NMR spectroscopy (Grasdalen et al., 1979). Since it has been shown that abalone hepatopancreas extract contains a mannuronate-specific enzyme (Haugen et al., 1990), the abalone enzyme was used as source of mannuronate lyase for the control experiment. As shown in Table 4, cloned ALY degraded D-mannuronate and intact alginate to the same degree. However, enzyme activity using L-guluronan as substrate was only 25% of that when D-mannuronate was the substrate. The abalone enzyme actively degraded D-mannuronate, but showed less activity on D-guluronan. No trace of ALY activity was observed in *E. coli* JM109 carrying pUC18 (data not shown). Almost all other purified ALY enzymes reported to date have a strict substrate specificity for either mannuronate or guluronate, e.g. the mannuronate-specific ALY from a marine bacterium (Brown et al., 1991) and the guluronate-specific ALY from *Klebsiella* (Caswell et al., 1989). In neither case was the nucleotide sequence for the corresponding genes reported. Since the nucleotide sequence derived from the insert in pAL205 encodes only one ALY, we conclude that this ALY has dual substrate specificity with a preference for mannuronan over guluronan.

We thank Dr T. Muramatsu (Nagasaki Univ.) for kindly providing us with his preparation protocol of uronate homopolymers, Dr S. Kuramitsu (Osaka Univ.) for aspartate aminotransferase assay, and Dr S. Sakada (Osaka Univ.) for NMR spectroscopy analysis. We also thank Dr R. L. Rodriguez (University of California, Davis) for critical reading of the manuscript and for making useful and helpful suggestions. We would like to thank Dr S. F. Sasaki (Tokai Univ.) for her helpful advice and encouragement, and Dr T. Seki and M. Kataoka for their useful suggestions. We are grateful to F. Sawazumi and H. Miyazaki (ICBiotech, Osaka Univ.) for their support, and to the staffs of Applied Biosystems Japan for determining the N-terminal amino acid sequence of the protein. This work was in part supported by Komori Memorial Foundation.

### References


