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

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A gene (*aly*) encoding alginate lyase (ALY; EC 4.2.2.3) was isolated from a library constructed with the cosmid vector pHCT9 and *Sau*3AI-digested genomic DNA of *Pseudomonas* sp. OS-ALG-9. Successive subcloning of the *aly*-containing cosmid enabled us to locate the gene on a 2.3 kb *Hpa*I 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 *Pseudomonas* sp. OS-ALG-9 was found in the amino acid sequence deduced from this ORF between nucleotides 282 and 366. Expression of *aly* was induced by IPTG in *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 α -L-guluronate (G) and β -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 *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 & Sherbrock-Cox, 1981), marine molluscs (Nakada & Sweeny, 1967; Nishizawa *et al.*, 1968) and seaweeds (Madgwick *et al.*, 1973; Shiraiwa *et al.*, 1975). Most alginate lyase (ALY)

enzymes, however, have been purified and characterized from bacteria (Davidson *et al.*, 1976; Romeo & Preston, 1986; Lange *et al.*, 1989). These were found to be lyases instead of hydrolases, catalysing the β -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 *Klebsiella pneumoniae* has been cloned and expressed in *Escherichia coli* (Caswell *et al.*, 1989). This was followed by the cloning and expression in *E. coli* of the gene for a mannuronate-specific ALY from a marine bacterium (Brown *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, *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 *et al.*, 1991). In this paper, we report the nucleotide sequence of a gene, designated *aly*, for ALY from *Pseudomonas* sp. OS-ALG-9. The data provide new insights into the structure–functions relationship of bacterial ALY.

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Abbreviations: ALY, alginate lyase; *aly*, gene encoding ALY.

The nucleotide sequence data reported in this paper have been submitted to DDBJ and have been assigned the accession number D10336.

Table 1. *Bacterial strains and vectors*

Strain or vector	Description	Source or reference
Bacterium		
<i>Pseudomonas</i> sp. OS-ALG-9		Kinoshita <i>et al.</i> (1991)
<i>E. coli</i> LE392	F ⁻ <i>hsdR514(r⁻m⁺)supE44 supF58 galK2 galT22 metB1 trpR55 lacY1</i>	Sambrook <i>et al.</i> (1989)
HB101	<i>supE44 hsdS20(r_B⁻m_B⁻) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 ml-1</i>	Boyer & Roulland-Dussoix (1969)
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F'[traD36 proAB⁺ lacI^q lacZΔM15]</i>	Yanisch-Perron <i>et al.</i> (1985)
Plasmid or phage		
pHC79	Ap ^R cosλ	Hohn & Collins (1980)
pBR322	Ap ^R Tc ^R	Bolivar <i>et al.</i> (1977)
pUC18	Ap ^R	Yanisch-Perron <i>et al.</i> (1985)
M13mp18		

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 & Doly (1979). Routine tasks such as restriction endonuclease digestion, ligation and agarose gel electrophoresis were carried out according to Sambrook *et 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 50 mM-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 0–40% (w/v) sucrose gradient 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 *Bam*HI and dephosphorylated with calf intestine alkaline phosphatase. The resulting concatemers were packaged *in vitro* 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 Gacesa & Wusteman (1990). Each transductant was transferred onto an LB-agar plate with ampicillin and 1 mg sodium alginate ml⁻¹, and incubated overnight at 37 °C. After growth was observed, 100 mg cetylpyridinium chloride ml⁻¹ was overlaid 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 1.25 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 (A₂₈₀)⁻¹]. The enzyme assays were reproducible ± 25%.

Homopolymeric block regions of D-mannuronate and L-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 *Hpa*I fragment of *aly*) were used to prepare deletions for locating *aly* and for nucleotide sequencing. Unidirectional deletions of the 2.3 kb *Hpa*I insert were created by *Bam*HI–*Kpn*I cleavage of the pUC18 portion of pAL20 and pAL12 followed by *exo*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.

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_{280})⁻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).

as previously described by Kinoshita *et al.* (1991). The enzyme was separated on SDS-PAGE (Laemmli, 1970) and transferred to PVDF membrane (Bio-Rad) by electroblotting. The N-terminal amino acid sequence of blotted protein was determined with an Applied Biosystems protein sequencer model 473A.

In vitro transcription/translation of *aly*. An *in vitro* transcription/translation kit (Amersham) was used according to the manufacturer's instructions. The samples of [³⁵S]methionine-labelled protein were separated on SDS-PAGE (12.5%, Daiichi Pure Chemicals). The gel was fixed in 10% (v/v) acetic acid, washed in water, soaked in EN³HANCE (Du Pont), washed again in water, dried under vacuum and exposed to X-ray film (Fuji).

Results and Discussion

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

Using the cosmid vector pH79, 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 (Fig. 1b) 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-

CCTATCTACACATGTGGTTTACCGATGCTGGGAAGCTTATGGCGCAACGGCACCTGGAC	60	GGGACGGTTACTTTTAGGCACGGCAACAATCCGAAGTTAGCAACAATTATTTATCGCA	1020
AGGTGTGGTAAGCATTAGCTCGGCAGTGAAGTCAACAGGATTGGCCACTACTTATGAGGA	120	GlyThrValThrPheArgHisGlyAsnAsnSerGluValSerAsnAsnTyrPheIleAla	
AGCGCTTGCTTTTATGATAATCTATATTAATAATATTTAAGATGAAATGAATTTTAT	180	AATAATGTAACCAATAGCGGTGGGTACGTATTATAGCGCAAAATCAAAAGTATATGGC	1080
1 GTTGGTTTGGTGTCTTATGCGGGTGCATTATAGCAGTTGTGCAAAAGCGGTGAAACG	240	287 AsnAsnValThrAsnSerGlyGlyValArgIleIleGlyGluAsnGlnLysValTyrGly	
7 ValGlyLeuValLeuLeuCysGlyLeuHisTyrSerSerCysAlaLysAlaValGluThr		AATTATTTGTACAAAGTGGCGGACGAACGTTGCGGTGGCAATTTCTGTGATGAATGCC	1140
GGCTCAAGTGCTGAAAAATAGAAAAACCAAGCAACCCTAAGCGAAAAACATACACCATC	300	307 AsnTyrLeuTyrLysValAlaGlyArgThrLeuArgSerAlaIleSerValMetAsnAla	
27 GlySerSerAlaGluLysIleGluAsnGlnAlaThrLeuSerGluLysThrTyrThrIle	360	TATGAAAAGCCCGCACTAAATGATTATGGCAGGTGAAAAATGCAGATATTCAAAACAAC	1200
AGCAGTCGCGCGAGCTCAGTCAATTAATTAATGCTGCTGATAAGTTCATCATGAAA	360	327 TyrGluLysProAlaLeuAsnAspTyrTrpGlnValLysAsnAlaAspIleGlnAsnAsn	
47 SerSerAlaAlaGluLeuSerGlnLeuAsnLeuMetProGlyAspLysValIleMetLys	420	347 IleIleValGlyAlaArgGluAlaPheValLeuGlySerGlyLysAspAsnAspArgThr	
67 SerGlyGluTrpLysSerGlnPheIleHisPheLysGlyLysGlyThrAlaGluLysPro	480	TTGGCACCTGATGGGGTTAATATCAGCAATAATACATCATTAACCAACAACGTTATTG	1320
ATTACATTAACTGCGAAACCAAGGAAGTGCTGCTACGGGAAATTCACCTCAAA	540	367 LeuAlaProAspGlyValAsnIleSerAsnAsnTyrIleIleAsnProThrThrLeuLeu	
87 IleThrLeuThrAlaGluThrLysGlySerValLeuLeuThrGlyAsnSerAsnLeuLys	600	387 ValThrGlnAspGluProLysAsnLeuLysMetGlnAsnAsnGlnValGluGlyAlaSer	
ATTGATGGTGAGTGGCTAGTGGTAGATGGCCTATCTTCAAAACGGGTTTTCCTCAAAA	660	ATTGTTACAGGTTTGTGAAAATGGGTAACTGACCTTACAAATGTCGGATGGGATTGGCAG	1440
107 IleAspGlyGluTrpLeuValValAspGlyLysPheLysAsnGlyPheSerLeuLys	720	407 IleValThrGlyPheValLysMetGlyAsnAspLeuGlnMetSerAspGlyIleTrpGln	
GATGATGTGGTGGTTTCCACCAAAACACCACGAATAGCGGTTTAAACCAATACCAATC	780	427 LysLysThrGluIleLysLysProPheTrpLeuAlaThrAlaIleGlyProGluTrpLys	
127 AspAspValValValPheThrLysThrThrAsnSerArgLeuThrAsnThrSerIle	840	447 LysAspHisArgSerPheIlePheLys***	
GAAAATTATAATCCCGTTGATAAACTTTAGACTATAAATGGGTGTCGCTTTATGGGCAT	900	GCGTCTATTCCGTCATTTGACGATAGGAGAAATCTTAAAGCTCAGAGGATAGTGCACA	1620
147 GluAsnTyrAsnProValAspLysThrLeuAspTyrLysTrpValSerLeuTyrGlyHis	960	AAAACTGCCCCATAGCCCCGACAGTAGTGAGCAGAGTTGGCAAGCTTAGCTTGCCAAAC	1680
CATACCGAGTGGATCATTGCAGTATCAGGAGAAAAACCATCAGGGAACCTACTTTGGTG		GAGTAAACGAATGGCGGGACTTTCCGTTGGCCCCCTGCCTAAAAATGGCGCTCCTAAAA	1740
167 HisAsnArgValAspHisCysSerIleThrGlyLysAsnHisGlnGlyThrThrLeuVal		ATCAATACACTTTTAACCTATAAAGCTTAACACGAATTAACACCTAAACCCCTTGCATGTT	1800
187 ValTrpLeuAspAspLysProAsnTyrHisGlnIleAspHisAsnTyrPheGlyProArg		TGTTTATTAGTTTCGTATATTACGATTATTTCGTAATTAAGTATGAGGAAATTAACAC	1860
CCAGAGTTGGGCGCAATGGAGGCGAAACCATCCGTATAGGAACAAGTGCTTTTCAATG		AGCAAGAGGAGAAAGCAATGCTAGCCCTTTGGCAGGTAGGTGCTGGTTTATTAAAGATT	1920
207 ProGluLeuGlyAlaAsnGlyGlyGluThrIleArgIleGlyThrSerAlaPheSerMet		TTATGGATGCCATGCCGAACCAAGACACCATATACCATTGTGCATCAACGATCAAAA	1980
AATGATTCTTATCGAAGCGTGCAGAACAAATATTTTGAATAATGCGATGGCAGGTAGAG		ATTTAGAGCGT	1991
227 AsnAspSerTyrArgThrValGlnAsnAsnIlePheAspLysCysAspGlyGluValGlu			
ATCATTTTCGATCAAACTGGTTTATAAAATTTGAATAACCTTTTCTACGAATGTGCG			
247 IleIleSerIleLysSerGlyPheAsnLysIleLeuAsnAsnLeuPheTyrGluCysAla			

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 coordinates are provided at the right of the sequence and the amino acid residues are provided at the left starting from the first Met codon. Unique restriction sites *Eco*T14I (775–780) and *Bst*EII (1320–1326) 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 –35 and –10 regions are underlined in the 5' flanking region. The potential Shine–Dalgarno sequence is marked with dots below the DNA sequence.

containing cells was unaffected by IPTG (data not shown). This suggests that there is a promoter-like sequence in the pseudomonad insert in pAL12 and that *aly* might be expressed under the control of this sequence. On the other hand, *aly* in pAL20 is subject to *lacZ*p control. As shown in Fig. 1(b), deletion mutants of the 2.3 kb *Hpa*I insert of pAL20 and pAL12 were created. The resulting deletion derivatives, pAL205 (300 bp deletion) and pAL122 (600 bp deletion), both retained ALY activity, while the larger deletions carried in pAL203 and pAL123 resulted in loss of ALY activity (Fig. 1b). These results indicated that the region of *aly* essential for activity is located within approximately 1.4 kb (Fig. 1b). The cloned *Hpa*I fragment of pAL20 was hybridized to a single 4 kb *Bgl*II 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 *aly*

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' end of *aly* in these subclones is closer to the *lacZ*p of pUC18.

Nucleotide sequence of *aly*

Using a series of unidirectional nested deletions of pAL20 and pAL12, the nucleotide sequence of both strands of the *Pseudomonas* sp. DNA insert was determined (Fig. 2). The nucleotide sequence contains only one large ORF of 1365 bp, beginning with an ATG codon at sequence coordinates 163–165 (amino acid residue 1). To confirm that this ORF was responsible for conferring ALY activity to the host cells, two unique restriction sites *Eco*T14I and *Bst*EII, were used to introduce frame shift mutations. Filling in the cohesive

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 [35 S]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.

ends of either site with DNA polymerase I Klenow fragment resulted in the loss of ALY activity. Four sequences 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 σ^{70} -type or any other known prokaryotic RNA polymerase (Helmann & Chamberlin, 1988) could be observed in the 5'-noncoding 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 50 620 Da. The N-

terminal amino acid sequence of the enzyme purified from *Pseudomonas* sp. OS-ALG-9 cells was determined as N₂H-Glu-Lys-X-Tyr-Thr-Ile-Ser-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 46 361 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 [35 S]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 *Hpa*I 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-7 and *E. coli* JM109(pAL205)

The fractionation procedure and measurements of activities were as described in Methods.

Strain	ALY activity [milliUnits (ml culture) ⁻¹]	
	Extracellular	Intracellular
<i>Pseudomonas</i> sp. OS-ALG-9	5.9	5.2
<i>Escherichia coli</i> JM109(pAL205)*	10.3	117

* *E. coli* JM109(pAL205) was grown at 30 °C in the presence of 1 mM-IPTG.

Table 3. Effect of glycine addition on the leakage of ALY and AspAT from *E. coli* JM109(pAL205)

E. coli JM109(pAL205) was grown at 30 °C in 20 ml 2 × TY medium supplemented with ampicillin. After 5 h cultivation, expression of *aly* was induced with 1 mM-IPTG followed by enhancement of leakage of the gene product by adding 1 % glycine after 6 h. Enzyme activities of extracellular and intracellular fractions were measured.

Glycine addition	10 ¹² × ALY (milliUnits per cell)		10 ⁹ × AspAT (Units per cell)	
	Extracellular	Intracellular	Extracellular	Intracellular
–	2.74	28.6	–	1.88
+	33.3	42.3	0.501	1.43

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 ¹H 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-mannuronan and intact alginate to the same degree. However, enzyme activity using L-guluronan as substrate was only 25 % of that when D-mannuronan 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

Table 4. Enzyme activity toward D-mannuronan, L-guluronan and alginate of ALY from *E. coli* JM109(pAL205), abalone hepatopancreas extract and *E. coli* JM109(pUC18)

Enzyme activity towards substrates was measured as described in Methods. D-Mannuronan and L-guluronan were prepared for this study using the method of Haug *et al.* (1967). The abalone hepatopancreas extract was purchased from Sigma. ND, No detectable activity.

Substrate	Enzyme activity (milliUnits)		
	<i>E. coli</i> JM109 (pAL205)	Abalone extract	<i>E. coli</i> JM109 (pUC18)
Alginate	4.02	2.90	ND
L-Guluronan	1.00	0.712	ND
D-Mannuronan	3.64	3.87	ND

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.

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References

- BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513–1523.
- BOLIVAR, F., RODRIGUEZ, R. L., GREENE, P. J., BELTACH, M. C., HEYNEKER, H. L., BOYER, H. W., CROSA, J. H. & FALKOW, S. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95–113.
- BOYER, H. W. & ROULLAND-DUSOIX, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *Journal of Molecular Biology* **41**, 459–472.
- BROWN, B. J., PRESTON, J. F. & INGRAM, L. O. (1991). Cloning of alginate lyase (*algM*) and expression in *Escherichia coli*. *Applied and Environmental Microbiology* **57**, 1870–1872.
- CASWELL, R. C., GACESA, P., LUTRELL, K. E. & WEIGHTMAN, A. J. (1989). Molecular cloning and heterologous expression of a *Klebsiella pneumoniae* gene encoding alginate lyase. *Gene* **75**, 127–134.
- CLEWELL, D. B. & HELINSKI, D. R. (1969). Supercoiled circular DNA–protein complex in *Escherichia coli*: purification and induced

- conversion to an open circular form. *Proceedings of the National Academy of Sciences of the United States of America* **62**, 1159–1166.
- DAVIDSON, I. W., SUTHERLAND, I. W. & CHRISTOPHER, J. L. (1976). Purification and properties of an alginate lyase from a marine bacterium. *Biochemical Journal* **159**, 707–713.
- DERETIC, V., GILLM, J. F. & CHAKRABARTY, A. M. (1987). Alginate biosynthesis; a model system for gene regulation and function in *Pseudomonas*. *Bio/Technology* **5**, 469–477.
- DOUBET, R. S. & QUATRANO, R. S. (1984). Properties of alginate lyases from marine bacteria. *Applied and Environmental Microbiology* **47**, 699–703.
- GACESA, P. & WUSTEMAN, F. S. (1990). Plate assay for simultaneous detection of alginate lyases and determination of substrate specificity. *Applied and Environmental Microbiology* **56**, 2265–2267.
- GRASDALEN, H., LARSEN, B. & SMIDSRØD, O. (1979). A p.m.r. study of the composition and sequence of uronic acid residues in alginates. *Carbohydrate Research* **68**, 23–31.
- HAUG, A. & LARSEN, B. (1962). Quantitative determination of the uronic acid composition of alginate. *Acta Chemica Scandinavica* **16**, 1908–1918.
- HAUG, A., LARSEN, B. & SMIDSRØD, O. (1967). Studies on the sequence of uronic acid residues in alginic acid. *Acta Chemica Scandinavica* **21**, 691–704.
- HAUGEN, F., KORTNER, F. & LARSEN, B. (1990). Kinetics and specificity of alginate lyase. Part I, a case study. *Carbohydrate Research* **198**, 101–109.
- VON HEIJNE, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Research* **14**, 4683–4690.
- HELMANN, J. D. & CHAMBERLIN, M. J. (1988). Structure and function of bacterial sigma factors. *Annual Review of Biochemistry* **57**, 839–872.
- HOHN, B. & COLLINS, J. (1980). A small cosmid for efficient cloning of large DNA fragments. *Gene* **11**, 291–298.
- IKURA, Y. (1986). Effect of glycine and its derivatives on production and release of β -galactosides by *Escherichia coli*. *Agricultural and Biological Chemistry* **50**, 2747–2753.
- KARMEN, A. (1955). A note on the spectrophotometry assay of glutamic-oxaloacetic transaminase in human blood serum. *Journal of Clinical Investigation* **34**, 131–133.
- KINOSHITA, S., KUMOI, Y., OSHIMA, A., YOSHIDA, T. & KASAI, N. (1991). Isolation of an alginate-degrading organism and purification of its alginate lyase. *Journal of Fermentation and Bioengineering* **72**, 74–78.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LANGE, B., WINGENDER, J. & WINKLER, U. K. (1989). Isolation and characterization of an alginate lyase from *Klebsiella aerogenes*. *Archives of Microbiology* **152**, 302–308.
- MADGWICK, J., HAUG, A. & LARSEN, B. (1973). Alginate lyase in the brown alga *Laminaria digitata* (Huds.) Lamour. *Acta Chemica Scandinavica* **27**, 711–712.
- NAKADA, H. I. & SWEENEY, P. C. (1967). Alginic acid degradation by eliminases from abalone hepatopancreas. *Journal of Biological Chemistry* **242**, 845–851.
- NISHIZAWA, K., FUJIBAYASHI, S. & KASHIWABARA, Y. (1968). Alginate lyases in the hepatopancreas of a marine mollusc, *Dolabella auricula* Solander. *Journal of Biochemistry* **64**, 25–37.
- PREISS, J. & ASHWELL, G. (1962). Alginic acid metabolism in bacteria. *Journal of Biological Chemistry* **237**, 309–316.
- ROMEO, T. & PRESTON, J. F., III. (1986). Purification and structural properties of an extracellular (1-4)- β -D-mannuronan-specific alginate lyase from a marine bacterium. *Biochemistry* **25**, 8385–8391.
- SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning. A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- SANGER, F., NIKLEN, S. & COULSEN, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 2192–2195.
- SHIRAIWA, Y., ABE, S., IKAWA, T. & NISHIZAWA, K. (1975). Alginate lyase activities in the extracts from several brown algae. *Botanica Marina* **18**, 97–104.
- WAINWRIGHT, M. & SHERBROCK-COX, V. (1981). Factors influencing alginate degradation by the marine fungi: *Dendryphiella salina* and *D. arenaria*. *Botanica Marina* **24**, 489–491.
- WEISSBACH, A. & HURWITZ, J. (1959). The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli*. *Journal of Biological Chemistry* **234**, 705–709.
- YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.