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Cloning, sequencing and overexpression in *Escherichia coli* of the alginate-lyase-encoding *aly* gene of *Pseudomonas alginovora*: identification of three classes of alginate lyases[**]

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Abstract

A gene of *Pseudomonas alginovora*, called *aly*, has been cloned in *Escherichia coli* using a battery of PCR techniques and sequenced. It encodes a 210-amino-acid alginate lyase (EC 4.2.2.3), Aly, in the form of a 233-amino-acid precursor. *P. alginovora* Aly has been overproduced in *E. coli* with a His-tag sequence fused at the C-terminal end under conditions in which the formation of inclusion bodies is avoided. His-tagged *P. alginovora* Aly has the same enzymic properties as the wild-type enzyme and has the specificity of a mannuronate lyase. It can be purified in a one-step procedure by affinity chromatography on Ni^{2+}-nitriloacetate resin. The yield is of 5 mg of enzyme per litre of culture. The amplification factor is 12.5 compared with the level of production by wild-type *P. alginovora.* The six alginate lyases of known primary structure fall into three distinct classes, one of which comprises the pair *P. alginovora* Aly and *Klebsiella pneumoniae* Aly.

Abbreviations used : Aly-[His-tag], His-tagged *P. alginovora* alginate lyase Aly; HCA, hydrophobic cluster analysis; NTA, nitriloacetate.

INTRODUCTION

Alginates are linear uronic acid polymers in which *β*-D-mannuronate and α-L-guluronate residues are linked to form blocks of polymannuronate, polyguluronate and random sequences [1]. Alginates are synthesized as cell-wall polysaccharides by brown seaweeds and as exopolysaccharides by bacteria. Bacterial alginates are mainly of the polymannuronate type, with *O*-acetyl groups at the 2- and/or 3-position of D-mannuronate [2]. Alginate facilitates the attachment of mucoid *Pseudomonas aeruginosa* strains to the tracheal epithelium and respiratory mucins, protects the bacteria from phagocytes and prevents antibiotic uptake [3-5]. Consequently it is a major pathogenic factor in cystic fibrosis patients [1,6].

Alginate lyases have been isolated from various sources [7,8]. They catalyse the breakdown of alginate by a *β*-elimination mechanism, causing formation of a double bond between C-4 and C-5, 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 [7,9]. The primary structures of five alginate lyases are known. A1-I of a *Sphingomonas* sp. (converted into A1-II and A1-III by a self-processing reaction) depolymerizes non-acetylated and acetylated alginates [10,11]. AlxM of the marine bacterium A.T.C.C. 433367 [12], ALY of *Pseudomonas* sp. OS-ALG-9 [13] and AlgL of *P. aeruginosa* [14,15] are each mannuronate lyases. Aly of *Klebsiella pneumoniae* subsp. *aerogenes* is a guluronate lyase [16]. Three alginate lyases have been overproduced [16-18]. The previously described AlxM was cleaved between two cysteine residues, Cys-169 and Cys-183, themselves linked by a disulphide bridge [12]. It has been overproduced in *Escherichia coli* as an unnicked polypeptide chain referred to as $AlxM_B$ [18].

[**] This paper is dedicated to the memory of Professor Georges Michel.

The nucleotide sequence of *P. alginovora aly* is deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under accession no. X83679.

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As reported previously [19], *Pseudomonas alginovora* is an alginate lyase producer. The present paper describes the purification of a *P. alginovora* alginate lyase, Aly, and the cloning and sequencing of the encoding gene, *aly*. It defines the conditions under which the enzyme can be overproduced in *E. coli* and purified in a one-step procedure. It also identifies three distinct classes of alginate lyases.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides and culture conditions

P. alginovora strain XO17, CIP 102941, was maintained at 25 °C on sea water nutrient agar plates (Difco Laboratories). It was grown at 25 °C in Zobell Medium as described by Boyen et al. [19].

E. coli Max Efficiency DH5α (Gibco BRL, Life Technologies, Inc.), *E. coli* Top 10 F' (Invitrogen, Leek, The Netherlands), lysogenic *E. coli* BL21 (DE3) and *E. coli* HMS174 (DE3) (Novagen) were used as hosts for recombinant plasmids. The *E. coli* transformants were grown at temperatures ranging from 18 to 37 °C in Luria-Bertani (LB), Terrific Broth (TB) [20] and M9H [21] media containing 50 μg of ampicillin and 30 μg of chloramphenicol/ml. pUC18 (Gibco BRL) was used to subclone DNA fragments and to prepare double-stranded DNA for sequencing. pET-22b (for the expression of recombinant proteins and conferring ampicillin resistance) and plysS (for the stabilization of target genes and conferring chloramphenicol resistance) were from Novagen. Oligonucleotides were synthesized by Eurogentec, Liège, Belgium.

Alginate lyase plate assay

Alginate lyase-producing *E. coli* strains were identified by growing them at 28 °C on 'black medium' plates containing 0.5% agar, 0.5% yeast extract (Difco), 1 % high-viscosity alginate of *Macrocystis pyrifera* (Sigma, St. Quentin Fallavier, France), 0.5% active charcoal (Serva, Heidelberg, Germany), 50 μg/ml ampicillin and 30 μg/ml chloramphenicol [22].

Alginate lyase activity

The substrates were low-viscosity, acetyl-group-free, alginates from *Macrocystis pyrifera* (60% mannuronate; Sigma) and *Laminaria hyperborea* (44% mannuronate; Algocean, Landernau, France) and low-molecular-mass mannuronate and guluronate polymers, each containing 15-20 saccharide units (gifts from Dr. A. Heyraud, CERMAV, Grenoble, France). Enzyme solutions (400 μl) containing 0.3 % substrate in 225 mM Tris/HCl, pH 7.5, or 75 mM sodium phosphate (pH 7.5)/ 450 mM NaCl were incubated at 25 °C for 5 min. The unsaturated non-reducing groups produced by β-elimination were measured using 3-deoxy-D-manno-octulosonic acid (up to 50 nmol) as standard. The conditions described in Karkhanis et al. [23] were modified as in Malissard et al. [18]. One unit of enzyme activity produced 1 μmol of non-reducing, unsaturated, terminal group per min.

Production, purification, trypsin degradation and amino acid sequencing of wild-type *P. alginovora* Aly

The enzyme was produced and purified as described by Boyen et al. [19]. *P. alginovorα* XO17 was grown in 5 litres of Zobell medium supplemented with 0.1% sodium alginate from *Lαminariα digitαtα* (Sobalg). The enzyme present in the culture supernatant was concentrated by ultrafiltration through 10 kDa cut-off membranes and purified by ammonium sulphate precipitation, gel filtration and anion-exchange chromatography.

SDS/PAGE (12% gels) of *P. αlginovorα* Aly was carried out according to Laemmli and Favre [24]. Gels were stained with PAGE Blue 83 or electroblotted for 2 h at 0.8 mA/cm^2 on an Immobilon membrane (Millipore), using a Pharmacia LKB Novablot apparatus [25]. Membrane, with electrotransferred Aly, was inserted into the sequencing chamber of an Applied Biosystems 473A amino acid microsequencer. Acrylamide slices containing about 40-50 μg of SDS/PAGE-purified Aly (stained with 0.003 % Amido Black) were submitted to proteolysis with 1 μg of trypsin in 150 μl of 0.1 M Tris/HCl (pH 8.8)/0.01 % Tween 20 for 5 h at 37 °C. Trypsin-degraded Aly was submitted to reverse-phase HPLC on a 250 mm × 4.6 mm μBondapak C18 column. Elution, carried out with an acetonitrile gradient from 2to45 % in 0.1 % trifluoroacetic acid at a flow rate of 0.2 ml/min, allowed many peptides to be separated. Among them, peptides P1 and P2 were eluted at 22 and 24 % acetonitrile respectively. They each were submitted to amino acid microsequencing in the 473A microsequencer. The amount of phenylthiohydantoin derivative of the first amino acid residue was about 210 pmol for both peptides. The N-terminal sequences of Aly and P2 were used to synthesize nucleotides O1 and O2 respectively (see the Results and discussion section).

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DNA preparation and nucleotide sequencing

Genomic DNA from *P. alginovoraα* was isolated as in Hopwood et al. [26]. Plasmids were extracted from *E. coli* cells by the alkaline lysis method [27]. Recombinant plasmids were constructed and agarose gel electrophoresis was performed according to Sambrook et al. [20]. Restriction enzymes (Gibco BRL; Eurogentec) and T4 DNA ligase (Boehringer, Mannheim, Germany) were used with the buffers provided and under the conditions recommended by the suppliers. DNA segments cloned into pUC18 were sequenced by the dideoxynucleotide chain termination method [28] using M13 universal and reverse oligonucleotides or synthetic oligonucleotides as primers. Denaturation of double-stranded DNA was performed according to Zhang et al. [29]. Sequencing reactions were carried out by using the T7 sequencing kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) with [^{35}S]dATP labelling (ICN Biomedicals) or the Autoread sequencing kit (Pharmacia LKB) with fluorescent primers or incorporation of fluorescent dUTP. In this latter case, electrophoresis was done with an A.L.F. DNA sequencer.

PCR amplification

Mixtures (100 μl) containing DNA template (1-2 μg), deoxynucleotide triphosphates (20 nmol each) and oligonucleotide primers (0.5-1 μg each) were treated with Vent® DNA polymerase (2 units) in Vent buffer (New England Biolabs, Leusden, The Netherlands) or with Goldstar® DNA polymerase (0.4-1 unit) in Goldstar buffer (Eurogentec)/4 mM $MgSO_4$. The reactions were carried out in a DNA Thermal cycler (Biometra ; Trio Thermoblock) for 30 cycles, each with a 1 min denaturation at 94 °C, a 1 min annealing at T_m-5 °C (where T_m is the melting temperature of the primers) and a 150 s extension at 72 °C. The final elongation step at 72 °C was for 10 min. The T_m values of the primers were calculated using Primer Analysis Software OLIGO version 3.4 (National Biosciences, Hamel, MN, U.S.A.). The PCR products were purified using the QIA quick-spin PCR purification kit (Qiagen, Westburg, Leusden, The Netherlands).

Purification of His-tagged *P. alginovora* alginate lyase Aly (Aly-[His-tag])

E. coli HMS8 cells were grown at 18 °C in 1 litre of TB medium containing 50 /tg/ml ampicillin and 30 μg/ml chloramphenicol, collected by centrifugation, suspended in 100 ml of 50 mM sodium phosphate (pH 8)/500 mM NaCl, frozen and thawed (allowing the resident T7 lysozyme to perform cell lysis), and then the preparation was incubated with 1.7 units/ml DNAse I (EC 3.1.21.1 ; Sigma) for 20 min at 20 °C. The lysate, containing 387 mg of total protein (specific enzyme activity 1.35 units/mg of protein in 225 mM Tris/HCl, pH 7.5) was collected by centrifugation at 40000 *g* for 20 min and loaded on a 1.12 cm × 10 cm Ni^{2+}-nitriloacetate (NTA) resin column (Qiagen) equilibrated with 50 mM sodium phosphate (pH 7.0)/500 mM NaCl. Inactive proteins were eliminated by washing the column first with the same buffer and then with 50 mM sodium phosphate (pH 6.0)/500 mM NaCl. The adsorbed alginate lyase was eluted at about 180 mM imidazole in the last buffer using a two-step linear gradient of imidazole from 0 to 150 mM (4.5 bed vols.) and from 150 to 500 mM (0.5 bed vol.) at a flow rate of 0.5 ml/min. The active fractions were stored at -20 °C. The proteins were estimated according to Bradford [30], using BSA as standard.

Anti-(alginate lyase) polyclonal antibodies and immunoblot assays

Polyclonal antibodies, raised against *P. alginovoraα* Aly or $AlxM_B$ [18] alginate lyases, were prepared by injecting 60-100 μg of each enzyme into rabbits (two pairs), first in complete Freund's adjuvant and then in incomplete Freund's adjuvant, at 21-day intervals. Sera were collected 57 days after the first injection, decomplemented at 56 °C for 30 min and filter-sterilized (0.45 μm). To purify the antibodies, the alginate lyases (100 μg) were each submitted to SDS/PAGE (12%) and electrotransferred. Membrane strips, stained with Ponceau S, were incubated at 4 °C for 16 h with 5 ml of the relevant antiserum and washed successively with 20 mM Tris/HCl (pH 7.5)/500 mM NaCl (TBS), with 3 % gelatin in TBS containing 0.05 % Tween 20 (TTBS) to remove non-specifically bound antibodies, and with 5 ml of 0.1 M glycine, pH2.5, to dissociate the bound antibodies. The glycine buffer was neutralized immediately with 1 M NaOH (approx. 200 μl), and the purified antibodies were stored at -20 °C.

ELISA assays (in duplicate) were performed essentially as described by Bourdenet et al. [31]. The microtitre plates (Micro ELISA Dynatech) were coated with 0.1 ml of a solution of 5 μg/ml alginate lyase. The ELISA titres were calculated by multiplying the reciprocal of a serum dilution within the linear portion of the absorbance curve (A_{490} 0.7-1.2) by the corresponding A_{490} value.

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Alginate lyases (0.2 μg) were submitted to SDS/PAGE (12%) in the presence of prestained SDS/PAGE standards (Bio-Rad Laboratories, Ivry-sur-Seine, France) and transblotted on to Immobilon membranes. The membranes were treated with 3 % gelatin in TBS to block their protein-binding capacity, washed with TTBS and treated with purified anti-AlxM_B antibodies (100-fold dilution) and anti-Aly antibodies (1000-fold dilution). Complexes were detected as purple-red bands using the Bio-Rad immunoassay kit, a goat anti-(rabbit IgG)-alkaline phosphatase conjugate (1:3000 dilution) and the reagents *p*-Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt.

pi values, homology searches and hydrophobic cluster analysis (HCA)

Experimental pI values were measured by agarose gel slab electrofocusing using Pharmalytes 3-10 (Pharmacia) under the conditions recommended by the manufacturer. Theoretical pI values were computed using GCG software [32]. A homology search through the PIR protein sequence data bank (version 32) was made using FASTA and TFASTA [33]. For HCA, sequences taken from various data banks were converted into HCA plots using the HCA-PLOT program from Doriane (Le Chesnay, France). In these plots the amino acid residues are represented by their standard one-letter code, except for proline, glycine, serine and threonine which are represented by ★, ♦, ⊡ and □ respectively. HCA scores were computed as described by Gaboriaud et al. [34] and Lemesle-Varloot et al. [35].

Table 1 *Amino acid sequencing data of P. alginovora Aly. Amino acid residues are numbered according to the sequence shown in Figure 2.*

Peptide	N-terminal amino acid sequence	Amino acid positions
Wild-type Aly	GSFNDISWTLENEDN	1-15
Tryptic peptide P1	IWSSSIEVGGEE	168-179
Tryptic peptide P2	IGAYQLTGGEGEFHV	185-199

RESULTS AND DISCUSSION

Gene cloning and sequencing: amino acid sequence of *P. alginovora* Aly

Wild-type Aly produced by *P. alginovora* was purified to homogeneity (yield 0.4 mg per litre of culture) and the trypsin-degraded polypeptides P1 and P2 were isolated as described in the Materials and methods section. Table 1 gives the N-terminal amino acid sequences of Aly and of polypeptides P1 and P2.

The strategy of gene cloning involved the generation of the PCR products X, Y, Z and W, as illustrated in Figure 1. The nucleotide sequence of *aly* and the translated amino acid sequence of *P. alginovora* Aly are shown in Figure 2.

Product X

The genomic *P. alginovora* DNA was used as template, and oligonucleotide O1 (encoding the heptapeptide F^3NDISWT^9 of Aly; Table 1) and oligonucleotide O2 [complementary to the E^{194}GEFH(V)^{199}-encoding sequence of peptide P2; Table 1] were used as primers. The reaction product X, identified by gel electrophoresis, was about 600 bp long. It was subcloned into pUC18 at *Sma*I and sequenced using the M13 universal and reverse primers and oligonucleotides O3 (nucleotides 752-774) and O4 (complementary to nucleotides 822-843). Product X encoded a 197-amino-acid polypeptide, the sequence of which was the Phe^3-Val^{199} sequence shown in Figure 2.

Product Y

The *Hin*dIII, *Dra*I, *Cla*I and *Eco*RV genomic libraries were used as templates in an inverse PCR [36] with oligonucleotide O5 (nucleotides 1046-1068) and oligonucleotide 06 (complementary to nucleotides 527-548) as primers. (In an inverse PCR, a recircularized DNA fragment is used as template to amplify a gene from an internal position, and the sense of the amplification from the primers is opposite to that of a direct PCR.) The reaction product Y (about 2000 bp long), obtained with the *Hin*dIII library, was subcloned into pUC18 at *Sma*I and sequenced with the M13 universal primer and oligonucleotides O7 (complementary to nucleotides 285-306)

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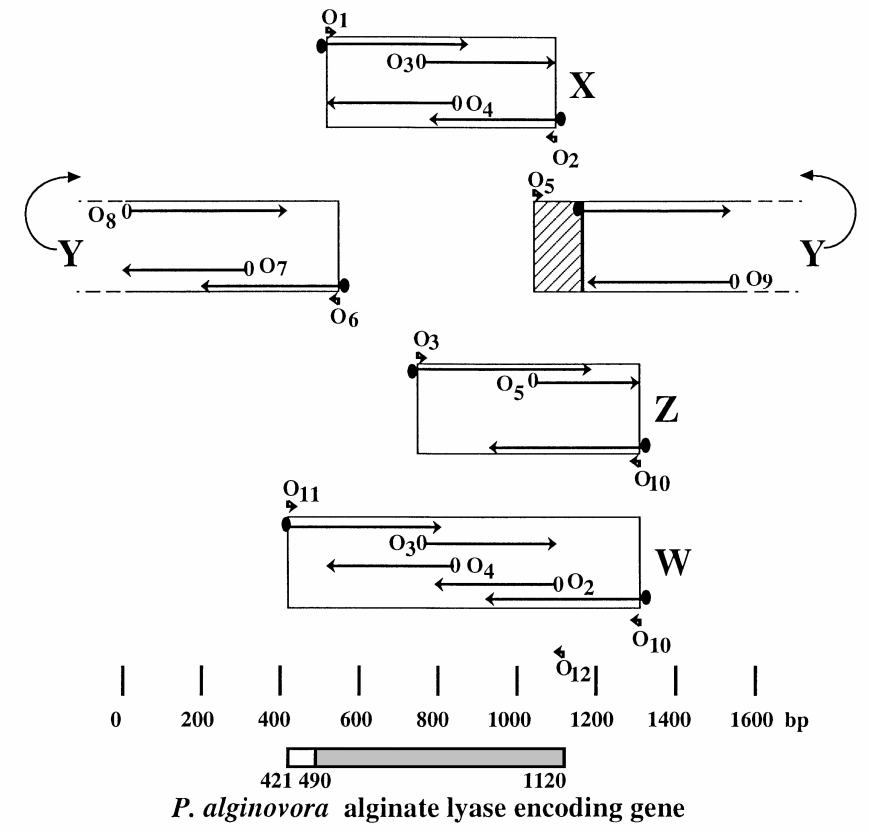
Status: Postprint (Author's version)

and O8 (nucleotides 1-22) (assay no. 1), and with the M13 reverse primer and oligonucleotide O9 (complementary to nucleotides 1519-1541) (assay no. 2). Assay no. 1 gave the nt 1-nt 548 sequence shown in Figure 2. The nt 422-nt 535 sequence encoded a 23-amino-acid signal peptide (Met^{-23} to Ala^{-1}) fused to the Gly^{1}-Asn^{15} sequence of the wild-type enzyme (Table 1). Assay no. 2, however, revealed that a fragment was deleted from nt 1046 as a result of the subcloning into pUC18.

Figure 1 *Strategy used for the cloning and sequencing of the P. alginovora alginate-lyase-encoding gene aly. Primers used for PCR amplification are indicated by → and ← ; sequences initiated with the M13 universal or reverse primers are indicated by ● ; and sequences initiated with synthetic nucleotides are indicated by O. The arrows indicate the orientation and length of the sequenced DNA segments. The unshaded box represents the signal-peptide-encoding sequence; the shaded box represents the mature alginate lyase-encoding gene. The hatched area in Y is a deleted segment (see the text).*



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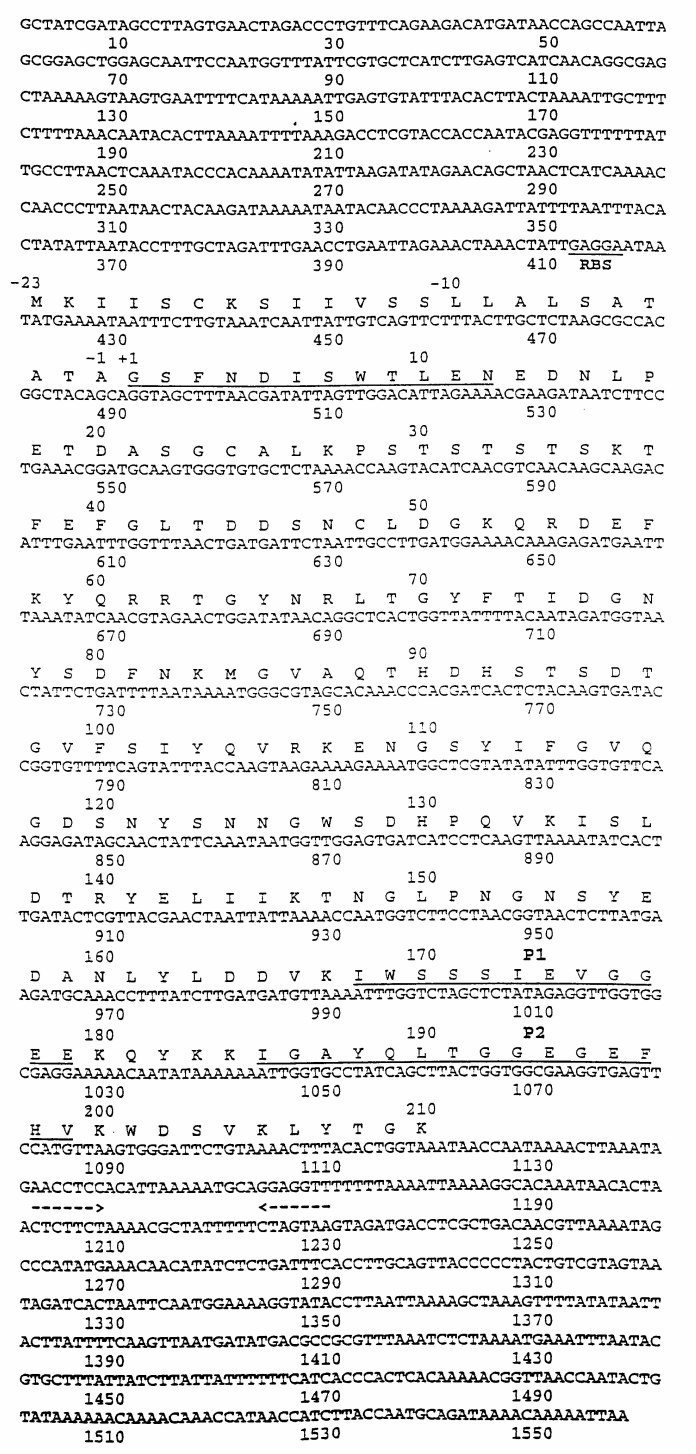
Status: Postprint (Author's version)

Figure 2 *Nucleotide sequence of P. alginovora aly and deduced amino acid sequence of its product Aly. The N-terminal amino acid sequences of the mature enzyme and the tryptic peptides P1 and P2 are underlined. Arrows indicate the putative stem-loop area. RBS, ribosome-binding site.*



Product Z

Product Z (about 600 bp long) was obtained with oligonucleotides 03 (as above) and O10 (complementary to nucleotides 1289-1309 with a *Pst*I site fused at the 5' end). Product Z was subcloned into pUC18 between *Sma*I and *Pst*I and sequenced in both directions with the M13 universal and reverse primers and oligonucleotide O5. It contained the nt 1046-nt 1170 segment that was missing from product Y. It also contained a TAA stop codon

starting at position 1121.

Product W

Products Y, X and Z arranged in an ordered sequence contained a 699-nucleotide open reading frame that started with a methionine-encoding ATG codon at position 422 and terminated with the TAA codon at position 1121. To check the accuracy of the sequence, the Goldstar and Vent DNA polymerases were each used to amplify the entire gene with oligonucleotide O10 (as above) and oligonucleotide O11 (nucleotides 422-449, with the Asp^{718} and *Nde*I sites fused in tandem at the 5' end). The two polymerases each generated the same product, W (approx. 900 bp long). Subcloning into pUC18 at the Asp^{718} and *Pst*I sites and sequencing confirmed the nucleotide sequence shown in Figure 2. Segments located upstream and downstream of *P. alginovora aly* had similarities with known *E. coli* consensus sequences. A putative GAGGA Shine-Dalgarno sequence occurred 5 bp upstream from the ATG initiation codon. The TATatT and TTGcCt sequences located 130 bp upstream from this Shine-Dalgarno sequence might correspond to the '-10' and '-35' regions of a promoter [37]. A putative stem-loop area located 18 bp downstream from the TAA stop codon might be a transcription termination site [38]. Moreover, this hairpin structure was followed by a TTTTT sequence, reminiscent of a Rho-inde-pendent termination site.

P. alginovora aly translated into a 210-amino-acid (Gly^{1}-Lys^{210}) protein preceded by a 23-amino-acid (Met^{-23}-Ala^{-1}) signal peptide (Figure 2). The theoretical molecular mass, 23 570 Da, of the signal-peptide-free protein coincided perfectly with the experimental value of 24 kDa derived from the migration of wild-type *P. alginovora* Aly on SDS/ PAGE. The theoretical (4.69) and experimental (5.20) pI values were similar. The tryptic peptides P1 and P2 occurred near the C-terminal end of the protein (Figure 2).

Overproduction, purification and properties of Aly-[His-tag]

To overexpress *aly* and facilitate purification of the overproduced enzyme, *Nde*I and *Xho*I sites were created by PCR at the 5' and 3' ends of a*ly* respectively using, as primers, the sense oligonucleotide O11 (as above) and the antisense oligonucleotide O12 (complementary to nt 1098-1120, with a *Xho*I site fused at the 5' end). The 719 bp PCR product was cloned into pET-22b between *Nde*I and *Xho*I sites. The resulting plasmid, pET-aly, contained *P. alginovora aly* with its own Met^{-23}-Ala^{-1} signal-peptide-encoding sequence and the His-tag (i.e. LEHHHHHH)-encoding sequence fused at the 3' end. The gene was under the control of the T7/*lac* operator. In consequence, its expression was inducible by isopropyl *β*-D-thiogalactoside.

pET-aly was used to transform *E. coli* Top 10 F' as described by Sambrook et al. [20]. The pair 'supercoiled pET-aly/plysS' was used to transform *E. coli* BL21 (DE3), yielding *E. coli* BL34, and *E. coli* HMS174 (DE3), yielding *E. coli* HMS8. The production of Aly-[His-tag] by the (ampicillin- and chloramphenicol-resistant) transformants was monitored by varying the growth conditions: the medium, the temperature, the time and the duration of the induction. The produced protein underwent aggregation into inclusion bodies at growth temperatures of 24 °C or higher. The optimal conditions for the production of the enzyme in a water-soluble form were: a temperature of 18 °C; initiation of induction by adding 1 mM isopropyl *β*-D-thiogalactoside at A_{600} = 1.3 (in the case of *E. coli* BL34 grown in M9H medium) or at A_{600} = 0.9 (in the case of *E. coli* HMS8 grown in TB medium); and a duration of induction of 7 h (*E. coli* BL34) or 17 h (*E. coli* HMS8).

As described in the Materials and methods section, *E. coli* HMS8 was grown in 1 litre of TB medium under optimal conditions, the cells were lysed by freezing and thawing and the enzyme solution (387 mg of protein; specific activity 1.35 units/mgin 225 mM Tris/HCl, pH 7.5) was purified by chromatography on a Ni^{2+}-NTA resin column (yield 5 mg of protein; specific activity 71 units/mg in 225 mM Tris/HCl, pH 7.5). The amplification factor of 12.5 (by reference to the yield of enzyme production by the wild-type strain) was lower than that obtained with other recombinant alginate lyases [16-18]. This relatively low amplification factor may be attributable to the low temperature at which the *E. coli* transformants must be grown to avoid the formation of inclusion bodies.

Aly-[His-tag] had the same N-terminal amino acid sequence (GSFNDISW) as *P. alginovora* wild-type Aly, showing that cleavage of the signal peptide occurred during synthesis in *E. coli.* As shown by SDS/PAGE (Figure 3A), Aly-[His-tag] was more than 90 % pure, and its apparent molecular mass was slightly greater than that of *P. alginovora* Aly (because of the presence of the additional His-tag octapeptide). The theoretical (5.35) and experimental (5.8) pI values coincided well.

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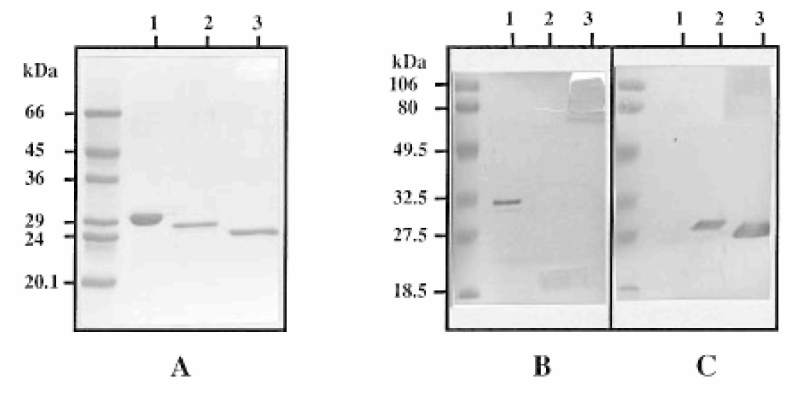
Figure 3 *SDS/PAGE (12% gels) of $AlxM_B$ (lanes 1), P. alginovora Aly-[His-tag] (lanes 2) and P. alginovora Aly (lanes 3). Coomassie Blue staining is shown in (A). Western immunoblots are also shown using antisera directed to $AlxM_B$ (B) and Aly (C). All samples were solubilized in SDS/PAGE sample buffer at 100 °C for 5 min. Prestained proteins of standard molecular masses (kDa) are shown.*



In 225 mM Tris/HCl, pH 7.5, buffer (*I* 0.18) and at 25 °C, *P. alginovora* Aly and Aly-[His-tag] had identical degrading activities towards the alginate substrates tested (see the Materials and methods section) : 97 units/mg with a mannuronate polymer (15-20 units), 75 units/mg with *M. pyrifera* alginate (60 % mannuronate), 20 units/mg with *L. hyperborea* alginate (44 % mannuronate) and 7 units/mg with a guluronate polymer (15-20 units). At higher ionic strength (*I* 0.66), and in 75 mM sodium phosphate (pH 7.5)/450 mM NaCl buffer, the enzyme activity was decreased by 50 %. The conclusion that *P. alginovora* Aly is a mannuronate lyase and not a guluronate lyase is at variance with that proposed previously [19]. P. *alginovora* might produce a guluronate lyase in addition to Aly.

P. alginovora Aly as a member of a particular class of alginate lyases

The six alginate lyases of known primary structure (including *P. alginovora* Aly) have various molecular masses. The polypeptide chains (including the signal peptide) contain 413 residues *(Sphingomonas* A1-III), 398 residues (*Pseudomonas* ALY), 368 residues (*P. aeruginosa* AlgL), 307 residues (*K. pneumoniae* Aly), 285 residues (marine bacterium sp. AlxM) and 233 residues (*P. alginovora* Aly). Hence one important conclusion derived from the present study is that an ~200-amino-acid polypeptide can adopt the required folding topology for breaking glycosidic linkages by a β-elimination reaction.

Homology searches carried out by Baron et al. [16] using methods based on single amino acid properties revealed statistically insignificant or marginal similarities between ALY, AlgL, AlxM and *K. pneumoniae* Aly, except for the presence of a 9-amino-acid motif (YFKAGVYNQ) at positions 257-265 in AlxM and 274-282 in *K. pneumoniae* Aly, close to the C-terminal end of the polypeptide chains.

These four alginate lyases were re-examined by HCA, and the same method was applied to A1-III and *P. alginovora* Aly. HCA is a powerful method for comparing proteins that are weakly related in primary structure. It rests upon a duplicated representation of the amino acid sequences on an α-helical two-dimensional pattern in which the hydrophobic residues tend to form clusters. As the six amino acid residues adjacent to residue *i* are *i*-4, *i*-3, *i*-1, *i*+1, *i*+3 and *i*+4, distant information becomes visible more readily. Hydrophobic clusters of similar shapes, sizes and relative positions are associated with definite secondary structures. The extent of similarity between two amino acid sequences is expressed by a HCA score, as defined in [34,35]. HCA scores of 65 % are found among proteins having the same overall folding (but with significant structural differences), and values of 80% or more are found among proteins of super-imposable three-dimensional structures.

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Table 2 *Amino acid sequence classes of alginate lyases. The amino acid numbering includes the signal peptide. The numbers of amino acid identities and similarities were derived from the alignments shown in Figure 4.* ∑ *indicates the number of identities plus similarities, as a percentage. Pairwise HCA scores have been computed from the HCA plots, as described by Gaboriaud et al. [34].*

Alginate lyase	Sequence	Amino acid residues	No. of identities	No. of similarities	∑ (%)	HCA score
Class 1						
Sphingomonas sp. A1-III	Gln^{59}-Ala^{407}	349				
P. aeruginosa AlgL	Gln^{26}-Ser^{368}	343	91	80	49	
Sphingomonas sp. A1-III	Tyr^{136}-Ala^{330}	195				
P. aeruginosa AlgL	Me^{92}-Ala^{290}	199	60	53	56.8	69.9
Class 2						
Pseudomonas sp. ALY	Met^{1}-Lys^{183}	183				
Photobacterium sp.* AlxM	Met^{1}-Lys^{190}	190	35	55	47.4	67.4
Class 3						
K. pneumoniae Aly	Phe^{29}-Glu^{221}	193				
P. alginovora Aly	Phe^{26}-Glu^{219}	194	40	48	45.4	71.7

* A tentative assignment to the genus *Photobacterium* has been proposed for the marine bacterium A.T.C.C. 433367 [39].

The HCA plots of the alginate lyases and the amino acid sequence alignments derived from these plots are shown in Figure 4. The results of the analysis are summarized in Table 2. The following observations were made, (a) The extent of similarity between A1-III and AlgL is largely above the cut-off point on the basis of which one may safely assume similarity in the three-dimensional structures. These two alginate lyases fall into class 1. One may note, however, that the highest level of similarity resides within the ~200-amino-acid sequences that extend from Tyr^{136} to Ala^{330} in A1-III and from He^{92} to Ala^{290} in AlgL. (b) ALY, AlxM and the *K. pneumoniae* and *P. alginovora* Alys each contain a polypeptide segment also about 200 amino acids long which, upon pairwise comparison, allows two additional amino acid sequence classes to be defined. ALY and AlxM are of class 2, and the *K. pneumoniae* and *P. alginovora* Alys are of class 3. The HCA scores are about 70 %, which implies that the pairs of polypeptides share related folding topology. (c) Inter-class similarity is almost non-existent. As shown by Western blotting (Figures 3B and 3C), *P. alginovora* Aly does not react with anti-AlxM antibodies (titre 300; dilution 1:100), AlxM does not react with anti-(*P. alginovora* Aly) antibodies (titre 3000; dilution 1:1000), but *P. alginovora* Aly and its derivative Aly-[His-tag] react with the anti-Aly antibodies. (d) A conserved aspartic acid residue occurs at positions 179 in A1-III and 135 in AlgL, at positions 58 in ALY and 67 in AlxM, and at positions 67 and 70 in *P. alginovora* and *K. pneumoniae* Alys respectively. (e) With the exception of *P. alginovora* Aly, which is 233 amino acids long, the ~200-amino-acid polypeptide that appears to define the 'core' of the five other alginate lyases represents only part (sometimes less than half) of the amino acid sequence. The mannuronate lyase AlxM and the *K. pneumoniae* guluronate lyase Aly each have a conserved 9-amino-acid segment close to the C-terminal end of the protein. These observations suggest that, like many glycosyl hydrolases [40], the alginate lyases may be of modular design. (f) The proposed class grouping does not correlate with the substrate specificity of the alginate lyases. An increasing number of proteins are known that have similar folds but insignificant sequence similarities [41]. The alginate lyases of classes 1, 2 and 3 and, perhaps, the pectate lyases (which also cleave glycosidic linkages by a β-elimination reaction) may belong to the same superfamily and may have a common fold signature. These questions are still open. At the present time, only the pectate lyases PelC and PelE of *Erwinia chrysanthema* [42] and Pel of *Bacillus subtilis* [43] are of known three-dimensional structure. They each adopt the same fold topology of parallel β-strands wound into a large right-handed coil [44].

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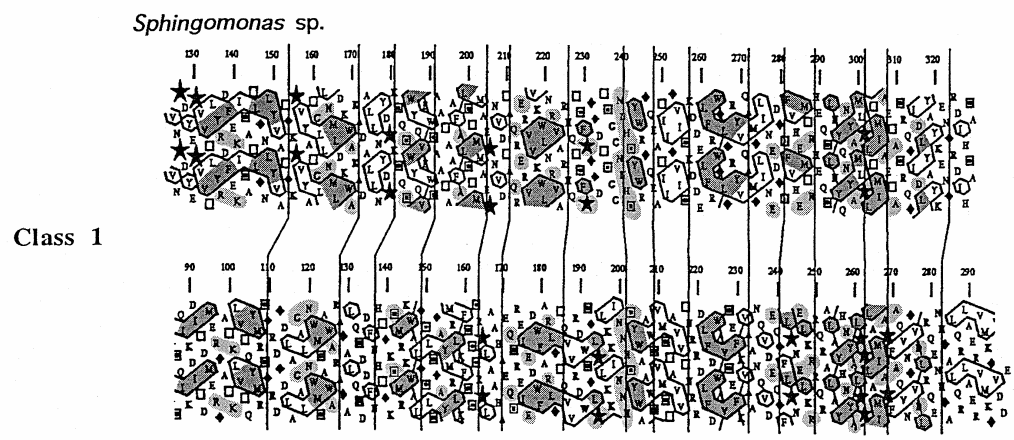
Figure 4 *HCA plots and deduced amino acid alignments of alginate lyases. In HCA plots, conserved hydrophobic amino acids and clusters are shaded in dark grey; conserved hydrophilic amino acids are shaded in light grey. Vertical lines show the proposed correspondences between the sequences. In the alignments, amino acid identities are indicated by vertical lines and similarities by asterisks. Sequences were taken from: PRF 2009330A (Sphingomonas sp. Al), SwissProt Q06749 (P. aeruginosa), DDBJ D10336 (Pseudomonas sp. OS-ALG-9), SwissProt P39049 (Photobacterium ATCC 433367), the present study (P. alginovora), and GenBank L19657 (K. pneumoniae).*




```
Sphingomonas sp  59 QAVVKDPTASYVDVKARRTFLQSGQLDDRLKAALPKEYDCTTEATPNPQQGEMVIPRRYLSG-NHGPVNPDYEPVVTL------YRDFEK 141
                    ||    |   |*   * |                  |    *  | | |  |  |* *|  | *      **    |*      * |*|*
P. aeruginosa    26 QAADLVPPPGYYAAVGER----------------KGSAGSCPAVPPPYTGSLVFTSKYE-GSDSARATLNVKAEKTFRSQIKDITDMER  97

Sphingomonas sp 142 ISAT-LG-NLYVA--TGK-PVYATCLLNMLDKWAKADALLNY-DP-KSQ-SWYQVEWSAATAAFALSTMMAE--PNVDT-A-Q-RERVVK 218
                      || |  * |*   *|* * *| | ||**  ||*  || *  |  **  |*  * *   **|* *   *    | *   | | || *
P. aeruginosa    98 -GATKLVTQ-YMR--SGRDGDIA-CALNWMSAWARAGAL-QSDDFNHIGKSMRKWALGSLSGAY-MRLKFSSSRP-LAAHAEQSRE-IED 178

Sphingomonas sp 219 WLNRVARHQT-S-FPG-GDTSCC-NNHSYWRGQEATII--GVISKDDELFRWGLGRYVQAMGLINEDGSFVH-EMIRHEQSLHYQNYAML 301
                    |* |**  |    * | *      |||||| *   ***  *|**   *|| |**  *  |   **|*| |*  |* |**  | | |||*
P. aeruginosa   179 WFARLGT-QVVRDWSGLPL--KKINNHSYWAAW--SVMSTAVVINRRDLFDWAVSEFKVAANQVDEQG-FLPNELKRRQRALAYHNYALP 262

Sphingomonas sp 302 PLIMIAETASRQGIDLYAYKENGRDI-HSARKFVFAAVKNPDLI-KKYASEPQD-TRAFKPG-RGDLNWIE-YQRARFGFADELGFMIVP 386
                    || |||  |  *|*||    ||   * * | ***  *| * * *  |   | || |  *|   *  * |*| |  | *    *
P. aeruginosa   263 PLAMIAPFAQVNGVDL--RQENHGALQRLAERVMK-GVDDEETFEEK-TGEDQDMID-LKVDNK--YAWLEPYC-ALYRCEPNACSRPKK 344

Sphingomonas sp 387 IFDP-----RTGGSGTLLAYKPQGAAAQAPVS 413
                      *|    | ||  | * *  *|*
P. aeruginosa   345 DREPFNSFRLGGEVTRV-FSREGGS------ 368
```

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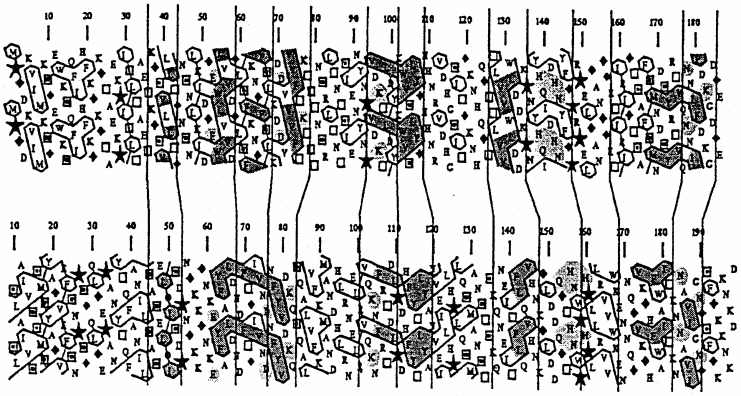
Class 2

Pseudomonas sp.



Photobacterium sp.

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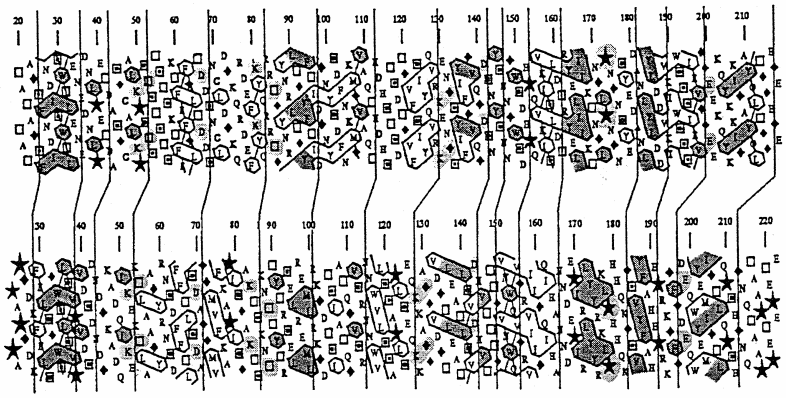
Pseudomonas sp      1 MPG-DKVIMKSGEWKSQFIHFKGKGTAEKP----ITLTAETK--------GS-VLLTGNSNLKIDG-EWLVVDGLSFKNGFSLKDDVVVF  75
Photobacterium sp   1 MIKSNLVI--SSLAIVSSMSYAGVEFSN-PSGQLGEPANYTQFANILSASELQIS-DPNGKKGNKEYFALDND-FT-GI-VNDNFYVD  81

Pseudomonas sp     76 TKTTTNS----RLTNTSI-ENYNPVDKTL--DY--KWVSLYGHNRVDHCSITGKNHQGTT----LVVWLDDKPNYH-QID--HNYFGPR 149
Photobacterium sp  82 -K-QSQALVFKMANDHLRNELR-VQKNFRIDLPDHFYTLYANVEILHPLQSMA----NSTSKQNEITFLQVH-NKGLDDQGIHNV--PH 160

Pseudomonas sp    150 PELGA----NGGETIRIGTSAFSMNDSYRTVQNN-IFDKC 184
Photobacterium sp 161 PLLRVVWKENNQGVK-GH--F-WA--ITK--NNAVICKG 191
```

Class 3

Pseudomonas alginovora



Klebsiella pneumoniae

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P. alginovora  26 FNDIS-WILENEDNLPETDASGCALKPSTSTSTSKTFEF-GLTDDSNCLDGKQRDEF-KYQRRIGYNR--LTGYFTIDGNYSDFNKMGVAQT 112
K. pneumoniae  29 F-ELSGWSLSVPVD-SDNDGKADQIKEKTLAAGYRNSDFFTLSDAGGWF-KAPISGAKTSKNTTYTRSELREMLRK-GDIS----IA-TQG 111

P. alginovora 113 HDHSTSDTGVFS----IYQVRKE----NGSYIFG-VQGDS-NYS-NNGWSDHPQVKISLDTRYE--------LTTKTNGLPNGNSYEDANLYLD-D 188
K. pneumoniae 112 VSR----NNWVLSSAPLSEQKKAGGVDGTLE-ATLSVDHVTTTGVN-W-QVG-RVTIGQIHANNDEPIRLYYRK--LPHHQKGS----VYFAHE 191

P. alginovora 189 -VKIWSSSIEVGGEEKQYKK-IGAYQL-------TGGEGE 219
K. pneumoniae 192 PRKG--------FG-DE-QWYEMIGTLQPSHGNQTAAPIE 221
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