ELSEVIER

www.elsevier.com/locate/carres

Carbohydrate Research 335 (2001) 11–21

CARBOHYDRATE RESEARCH

Cloning, sequence analysis and expression of *Pseudoalteromonas elyakovii* IAM 14594 gene (*alyPEEC*) encoding the extracellular alginate lyase

Tomoo Sawabe,[a,*] Hiromasa Takahashi,[a] Yoshio Ezura,[a] Peter Gacesa[b]

[a]*Laboratory of Microbiology, Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate 041-8611, Japan*

[b]*Faculty of Science and Engineering, The Manchester Metropolitan University, John Dalton Building, Chester Street, Manchester M1 5GD, UK*

Received 14 September 2000; received in revised form 6 June 2001; accepted 10 July 2001

Abstract

A gene (*alyPEEC*) encoding an alginate lyase of *Pseudoalteromonas elyakovii* IAM 14594 was cloned using the plasmid vector pUC118 and expressed in *Escherichia coli*. Sequencing of a 3.0kb fragment revealed a 1,197bp open reading frame encoding 398 amino acid residues. The calculated molecular mass and isoelectric point of the *alyPEEC* gene product are 43.2 kDa and p*I* 5.29. A region G^{165} to V^{194} in the AlyPEEC internal sequence is identical to the N-terminal amino acid sequence of the previously purified extracellular alginate lyase of *P. elyakovii*, and the calculated molecular mass (25.4 kDa) and isoelectric point (p*I* 4.78) of the region resembled those of the purified enzyme. Expression of enzymically-active alginate lyase from *alyPEEC* required growth of recombinant *E. coli* in LB broth containing 50% (v/v) artificial seawater (ASW). Alginate lyase activity with broad substrate specificity was detected in both 42 and 30 kDa products. Subcloning of the region G^{165} to N^{398} of AlyPEEC corresponding to the 30 kDa protein confirmed that this region of the *alyPEEC* gene encoded the active site of the enzyme. A region A^{32} to G^{164} corresponding to about 13 kDa of the N-terminal region of AlyPEEC showed about 30% identity to a putative chitin binding domain of *Streptomyces* chitinases, but did not exhibit any catalytic activity.

Keywords: Alginate lyase; Bacterial enzymes; Cloning; *Pseudoalteromonas elyakovii*; Sequence analysis

1. Introduction

Alginates are linear (1→4)-linked glycuronans comprised of residues of β-D-mannosyluronic acid (M) and its C-5 epimer α-L-gulosyluronic acid (G). These residues are arranged in block structures which can be homopolymeric [poly(β-D-mannosyluronic acid) (MM) and poly(α-L-gulosyluronic acid) (GG)] or heteropolymeric, i.e. containing random blocks (MG).[1] Enzymes that degrade alginate are invariably lyases rather than hydrolases[2] and are typically specific for either D-mannuronate- or L-guluronate-rich regions of the polysaccharide.[3] Alginate lyases catalyze the depolymerization of alginates by a β elimination mechanism with the formation of 4-deoxy-L-*erythro*-hex-4-ene pyranosyluronate at the nonreducing end of the resultant product.[2,3]

* Corresponding author. Fax: +81-138-405569.
E-mail address: sawabe@pop.fish.hokudai.ac.jp (T. Sawabe).

0008-6215/01/$ - see front matter
PII: S0008-6215(01)00198-7

Since the original description of alginate lyases more than 40 years ago,[4,5] more than 50 enzymes have been characterized[6] from a variety of microbial, animal and plant sources. Much of the earlier work on alginate lyases was directed towards using purified enzymes to analyze the fine structure of alginates and to aid the production of protoplasts from seaweeds. More recently, the emphasis has been focused on the structural–functional analysis of alginate lyases and elucidation of the biochemical pathways for the degradation and utilization of alginate by bacteria. Considerable progress has been made on the structural–functional analysis of alginate lyases with approximately 20 genes now cloned and sequenced.[6–16] This, in conjunction with preliminary three-dimensional structural data, has provided the basis for understanding the mechanism of action of alginate lyases.

In comparison to advances in the understanding of the mechanism of action of alginate lyases, relatively little is known about the alginate degradative pathways in bacteria or other organisms. The early work of Preiss and Ashwell[4] proposed that the degradation of alginates by a marine bacterium eventually generated monomers through the combined action of endo- and exo-enzyme activities and that these monomers were recycled through conventional metabolic pathways. More recently, work with the marine bacterium *Sphingomonas* has provided a more detailed insight into the possible pathway of alginate degradation.[9,17] *Sphingomonas* sp. strain A1, which was isolated from wastewater, appears to transport intact alginate into the cell where the polymer is effectively degraded into a series of oligosaccharides by endo-acting alginate lyases encoded by *aly* genes. These oligosaccharides are further degraded into monomers by an oligoalginate lyase which is encoded by the *oal* gene.[17]

Although the studies on *Spingomonas* are important, it is a non-marine bacterium and therefore the results may be of limited value in establishing the mechanisms for the degradation of marine brown algae, which are the major producers of alginates. The work on *Spingomonas* is complemented by our studies on the marine bacterium *Pseudoalteromonas elyakovii*,[18] which was isolated from a decaying thallus of *Laminaria*.[19] *P. elyakovii* is a pathogen of *Laminaria* that produces an extracellular alginate lyase[20,21] and a cell-bound polyM-specific degrading enzyme.[22] Interestingly, the extracellular enzyme is capable of degrading all block structures derived from sodium alginate and produces a series of tri- to octaoligouronates. This alginate lyase is novel in terms of its broad substrate specificity.[21] The cell-bound enzyme is also unique in that it specifically degrades polyM to produce saturated disaccharides.[22]

In addition to the metabolic studies, the enzymes from *P. elyakovii* have been used successfully to produce oligosaccharides with properties that are particularly useful in the preparation of specific food products.[23] Clearly, the ability to produce commercial quantities of these alginate lyases would facilitate the production of alginate oligosaccharides for applications in the food industry.

In this paper, we report the cloning, sequencing and expression of a broad substrate specificity extracellular alginate lyase from *P. elyakovii* IAM14594.

2. Results

Cloning of a gene encoding an alginate lyase.—About 7000 transformants carrying recombinant plasmids were screened using the plate assay, and four alginate lyase-producing *Escherichia coli* transformants were identified. All of the clones, pTPA1, pTPB1, pTPC1 and pTPD1, had a 3.0kb *Hin*dIII fragment. Although pTPA1, pTPB1 and pTPC1 had three, two and one additional *Hin*dIII fragments, respectively, subcloning experiments established that an alginate lyase gene was located on the common 3.0kb *Hin*dIII fragment. The *Hin*dIII fragment of pTPB3 has a *Pst*I, a *Sac*I site, two *Xba*I sites and two *Msp*A1I sites (Fig. 1). The restriction maps of the 3.0kb *Hin*dIII fragment derived from the other three clones were identical. The *Hin*dIII fragment was also subcloned into pUC18, and pTPB24 was constructed. The 1.9kb *Xba*I–*Hin*dIII, the 2.3kb *Pst*I–*Hin*dIII, the 0.9kb *Hin*dIII–*Sac*I and 1.6kb *Msp*A1I fragments were sepa-

rately subcloned into pUC18 or pUC118, to generate subclones pTPB11, pTPB15, pTPB18, and pTPB31, respectively. Only pTPB3 and pTPB31 showed alginate lyase activity (Fig. 1).

Determination and analysis of the gene sequence.—The nucleotide sequence and deduced amino acid sequence of pTPB31 are shown in Fig. 2. Translation of six possible reading frames revealed a complete open reading frame (1197bp), starting with an ATG codon at the position corresponding to 455–457 and terminating with a TAA codon corresponding to the position 1649–1651. The gene, designated as *alyPEEC*, is orientated in the same direction as the *lac* promoter of pUC18. Thirty amino acids G^{165} to V^{194} are identical to the N-terminal residue of the previously purified extracellular alginate lyase from *P. elyakovii* IAM14594.[21] The predicted product, AlyPEEC, of 398 amino acids residues M^{1} to N^{398} has a theoretical molecular mass of 43.2 kDa and p*I* value of 5.3, which are different from the purified enzyme which has a molecular mass of 32 kDa and p*I* value of 4.7.[21] Analysis of the N-terminal sequence of the extracellular alginate lyase of *P. elyakovii* indicates that the domain G^{165} to N^{398} of the open reading frame corresponds to

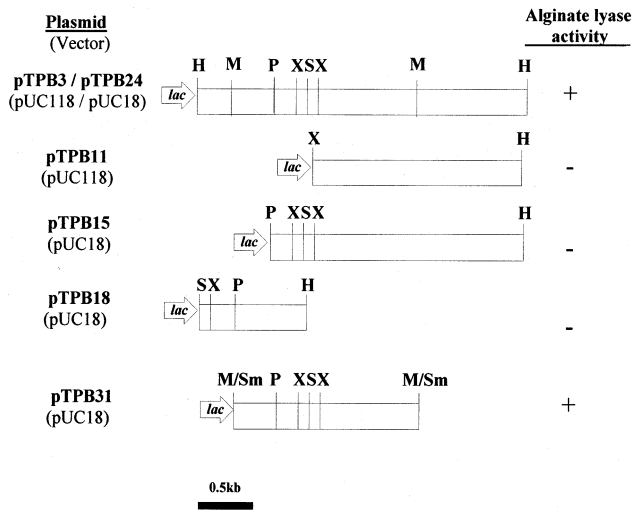

Fig. 1. Restriction map of *P. elyakovii* DNA clones. The alginate lyase activities of the transformants were detected by plate assay. Approximate fragment sizes: pTPB3, 3.0 kb; pTPB11, 1.9 kb; pTPB15, 2.3 kb; pTPB18, 1.0 kb; and pTPB31, 1.7 kb. The following restriction endonucleases were used: H, *Hin*dIII; P, *Pst*I; S, *Sac*I; X, *Xba*I; M/Sm, *Msp*AI1/*Sma*I (*Msp*AI1 produced blunt end).

the mature protein. The calculated values for this domain of 25.4 kDa and p*I* 4.8 are much closer to the experimental values for the mature protein. Determination of putative signal sequences using the PSORT program (see Experimental) indicated that the region M^{10} to A^{31} was a more likely candidate than M^{1} to A^{31}. It is predicted that the expressed protein is exported from the cell, through the outer membrane and into the medium.

Although the actual start codon is unknown, two possible ribosome-binding sites (RBS) were revealed (Fig. 2); one possible RBS, AAGA, may be located at 8bp upstream of an ATG codon (456–458) encoding M^{1} and another, GGAG, is located 11bp upstream of an ATG codon (480–483) encoding M^{10}. The −35 and −10 boxes of a putative gene promoter are located 72bp upstream of an ATG codon 456–458, the sequences are TTGttA and TAaAtT. A putative terminator region is located 16bp downstream of the stop codon. The terminator is an inverted repeat AAAGCCACTCAG and CTGAGTGGCTTT with a TTTAAAG loop. The existence of the promoter sites in *alyPEEC* was demonstrated by expression of enzyme activity with and without isopropyl-β-D-thiogalactopyranoside (IPTG) (see below).

Analysis of gene products.—Alginate lyase activity was detected in cell-free extracts of pTPB31 in LB broth containing 50% (v/v) artificial seawater (ASW) with or without IPTG (Table 1). Significantly less activity was present in the supernatants (Table 1). However, none or only trace activities were detected from cell-free extracts of cultures grown in LB broth. pTPB31 produced the greatest activity (1.32×10^{-3} U/OD280) among alginate lyase positive clones, pTPB3, or pTPB24 (data not shown). The specificity of the enzyme in the cell-free extract of pTPB3 was determined using a range of substrates. The activity for sodium alginate was 3.65×10^{-4} U/OD280, 2.35×10^{-4} U/OD280 for polymannuronate, 2.61×10^{-4} U/OD280 for polyguluronate, and 4.60×10^{-4} U/OD280 for MG random block; these activities were broadly similar. An activity staining method enabled the direct detection of *alyPEEC* gene products (Fig. 3). Cell-free extracts of pTPB24

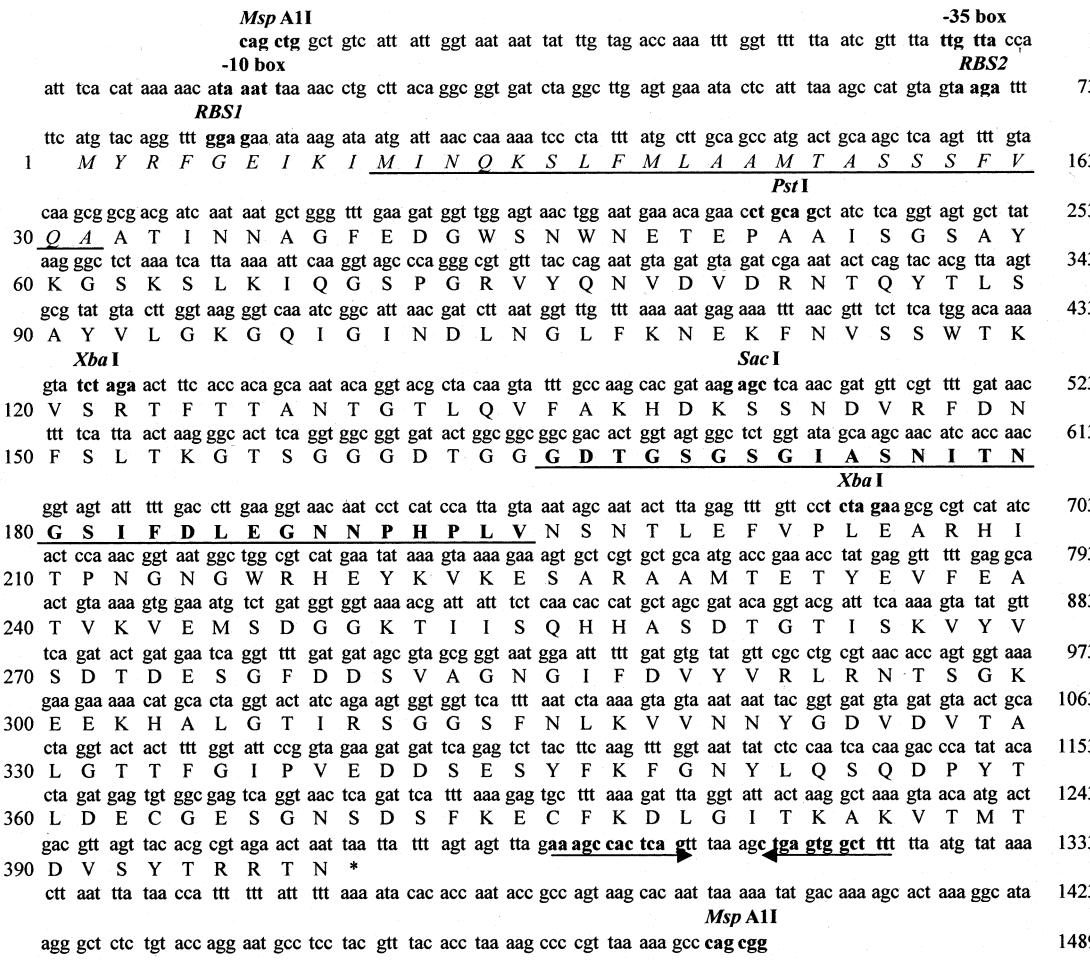

Fig. 2. Nucleotide sequence of *alyPEEC* and the deduced amino acid sequence. Promoter (-10 box and -35 box), ribosome binding sites (RBS1 and RBS2), terminator and restriction sites are indicated in bold. A putative terminator sequence is indicated by a pair of inverted arrows. Relevant restriction enzyme sites are labeled and indicated in bold. The 30-residue N-terminal amino acid sequence which is identical to the purified extracellular alginate lyase of *P. elyakovii* is indicated by bold and underlined. The possible signal sequence is indicated by italics and the most probable sequence predicted by the PSORT program is underlined.

Table 1
Expression of alginate lyase activity of the pTPB31

Culture medium [a]		Specific activity (U/OD280) [b]	
Composition	IPTG (mM)	Supernatant	Cell-free extract
50%ASW-LB	5	4.40×10^{-4}	1.32×10^{-3}
50%ASW-LB	0	7.37×10^{-6}	1.27×10^{-3}
LB	5	ND [c]	ND
LB	0	ND	3.62×10^{-6}

[a] All culture broth contained 100 μg/ml ampicillin.

[b] Alginate lyase activity was measured by TBA method. 1U is defined as the activity producing 1 μmol β-formylpyruvate. The reaction buffer contained 0.1 M Tris–HCl, pH 7.5 and 0.1% sodium alginate.

[c] Not detected or $< 1 \times 10^{-6}$ of the activity.

and pTPB31, and a supernatant sample of pTPB31 grown in LB broth containing 50% (v/v) ASW and 5 mM IPTG were applied to SDS–PAGE after mild denaturation at 70 °C for 5 min. The proteins were renatured and alginate lyase activity detected using sodium alginate-containing gel overlays. Alginate lyase activities were detected at the corresponding molecular mass of 42 and 30 kDa from the cell-free extracts of pTPB24 and pTPB31 cultured in LB broth containing 50% (v/v) ASW (Fig. 3, right panel, lanes 2, 4, 7 and 9), and the supernatant from pTPB31 using LB broth containing 50% (v/v) ASW and 5 mM IPTG (Fig. 3, right panel, lane 6), but not in the samples cultured in LB broth alone (Fig. 3, right panel, lanes 3, 5, 8 and 10).

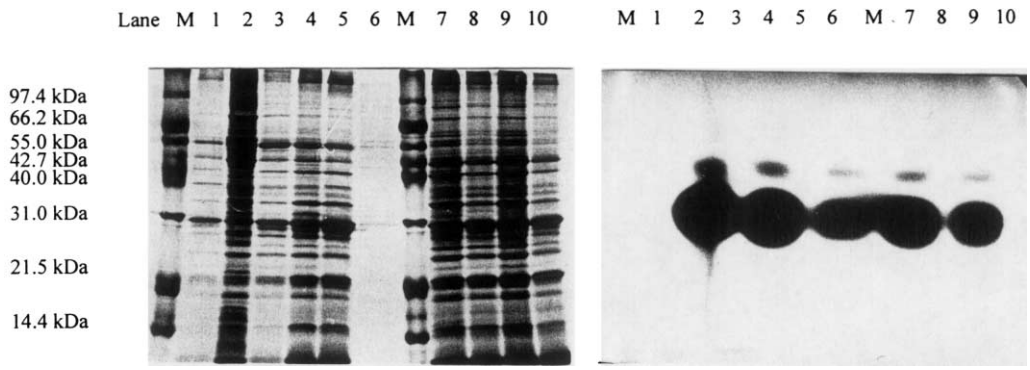

Fig. 3. Detection of alginate lyases expressed by transformants pTPB24 and pTPB31. Lane M; protein markers (phosphorylase B, 94.7 kDa; bovine serum albumin, 66.2 kDa; glutamate dehydrogenase, 55.0 kDa; ovalbumin 42.7 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa), lane 1; cell-free extract of *E. coli* containing pUC18 cultured in LB containing 5 mM IPTG, lane 2–5; cell-free extracts of pTPB31 cultured in LB broth containing 50% ASW with 5 mM IPTG (lane 2), without IPTG (lane 4), and cultured in LB broth with 5 mM IPTG (lane 3) and without IPTG (lane 5). Lane 6; supernatant of pTPB31 cultured in LB broth containing 50% ASW and 5 mM IPTG. Lanes 7–10; cell-free extracts of pTPB24 same as lane 2–5. The left-hand panel is the Coomassie Blue stained polyacrylamide gel, and right-hand panel is the activity stain.

The enzyme activity was weaker in the 42 kDa band than the 30 kDa band. The molecular mass of the 42 kDa gene products showing alginate activity was closely similar to the estimated mass of AlyPEEC A^{32} to N^{398}, and the 30 kDa was also similar to the domain G^{165} to N^{398} of AlyPEEC and the purified enzyme of *P. elyakovii*.

Localization of alyPEEC *gene products expressed in pTPB24 and pTPB31.*—Localization of AlyPEEC expressed in *E. coli* JM109 cells was determined using fraction specific marker enzyme activities. However β-galactosidase activity (the cytoplasmic marker) could not be detected in pTPB24 or pTPB31 because of the insertion of the *alyPEEC* gene into the *lacZ* gene. Prior to determining the localization of AlyPEEC, the fractionation procedure was tested using JM109 (pUC18) cultured in LB broth and LB broth containing seawater (Table 2). The marker enzymes, alkaline phosphatase and β-galactosidase, were detected in the periplasmic (PP) and cytoplasmic (CP) fractions, respectively, of JM109 (pUC18) cells cultured in either medium (Table 2). Using the same protocol, 84% of alginate lyase activity was detected in the PP fraction of pTPB24 (Table 2). In pTPB31, 84% of alginate lyase activity was detected in the culture supernatant (SP) fraction and 13% in the PP fraction (Table 2). No alginate lyase activity was detected in the CP fraction from either clone.

Each fraction was analyzed by the substrate containing gel overlay technique (Fig. 4). Protein bands with alginate degrading activity were observed in the PP fraction of pTPB24 (Fig. 4, lane 2) and the SP and PP fractions of

Table 2
Distribution of alginate lyase and marker enzyme activities expressed by clones

Clone/medium [a]	Fraction [b]	Distribution (%) [c]		
		AL	AP	β-Gal
pTPB24/LB+ASW	SP	9.6	25.3	ND
	PP	83.8	66.3	ND
	CP	6.6	8.4	ND
pTPB31/LB+ASW	SP	84.2	76.2	ND
	PP	13.4	14.9	ND
	CP	2.4	8.9	ND
JM109(pUC18)/LB +ASW	SP	NT	25.2	21.8
	CP	NT	70.1	3.3
	PP	NT	4.7	74.9
JM109(pUC18)/LB	SP	NT	22.1	14.8
	PP	NT	55.9	1.0
	CP	NT	22.0	84.2

[a] The transformants were incubated in LB or LB broth containing 50% artificial seawater (LB+ASW) with 100 μg/ml ampicillin, and 5 mM IPTG at 30 °C for 30 h.

[b] The method for fractionation was described in text. SP; supernatant, PP; periplasmic fraction, CP; cytoplasmic fraction.

[c] AL; alginate lyase activity, AP; alkaline phosphatase activity, β-Gal; β-galactosidase activity. NT; not tested, ND; not detected.

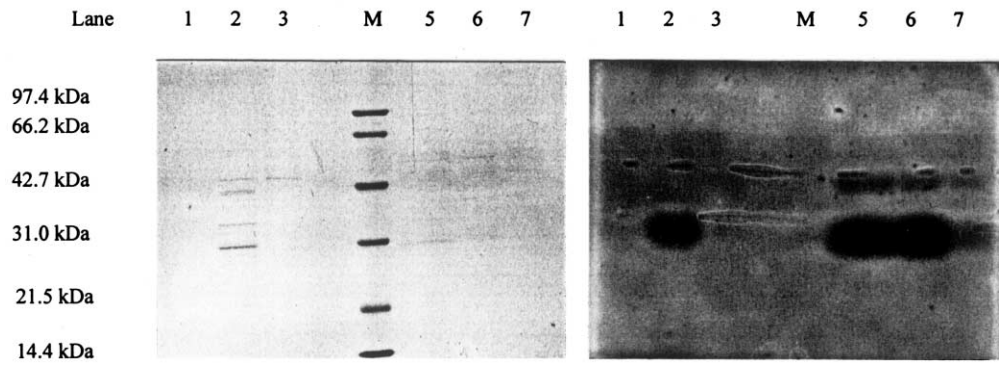

Fig. 4. Detection of alginate lyases activity in periplasmic and cytoplasmic fraction expressed by transformants. Lane M; protein markers (same as Fig. 3). Lanes 1–3; pTPB24 supernatant (lane 1), periplasmic (lane 2), and cytoplasmic (lane 3) fractions and lanes 4–6 pTPB31 supernatant (lane 4), periplasmic (lane 5), and cytoplasmic (lane 6) fractions. Left-hand panel is Coomassie Blue stained polyacrylamide gel, and the right-hand panel is the activity stain.

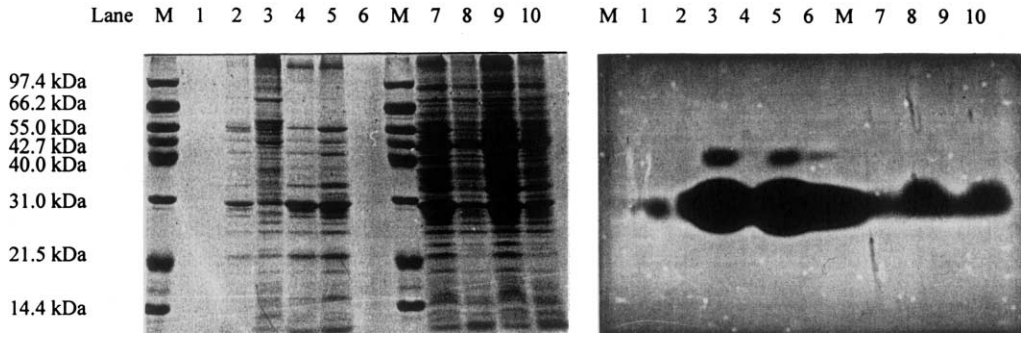

Fig. 5. Activity staining of the *alyPEEC*-mature region product. Lane M; protein markers (same as Fig. 3). Lane 1: culture supernatant of *P. elyakovii* IAM14594 with ZoBell 2216E containing 0.5% sodium alginate, lanes 2–5; cell-free extracts of pTPB31 cultured in LB containing 5 mM IPTG (lane 2) and without IPTG (lane 4), cell-free extracts of pTPB31 cultured in LB broth containing 50% ASW with 5 mM IPTG (lane 3), and without IPTG (lane 5). Lane 6: culture supernatant of pTPB31 grown in LB broth containing 50% ASW with 5 mM IPTG, lanes 7–10: cell-free extracts of pCD11 grown in LB broth with (lane 7) and without (lane 9) IPTG, cell-free extract of pCD11 cultured in LB broth containing 50% ASW with (lane 8) and without (lane 10) IPTG. Left-hand panel is Coomassie stained polyacrylamide gel, and the right-hand panel is the result of activity staining.

pTPB31 (Fig. 4, lanes 4 and 5, respectively). The estimated molecular mass of the enzyme as detected by activity staining was approximately 30 kDa. In addition, a faint spot of about 42 kDa molecular mass was observed in the SP and PP fraction of pTPB31.

Expression of AlyPEEC-mature region and sequence similarity analysis of AlyPEEC.— The region of *alyPEEC* from G^{165} to the stop codon, which was predicted to correspond to the mature 30 kDa enzyme was amplified and cloned into the expression vector pTrcHisB. A clone, pCD11, with alginate degrading activity was obtained. Activity was detected only in the cell free extract cultured with LB broth containing seawater (Fig. 5, lanes 8 and 9), and the specific activity was 6.0×10^{-5} U/protein, which was slightly lower than that obtained from pTPB24 and pTPB31. The gene product pCD11 corresponded to the fully processed derivative of AlyPEEC and was determined to be a single protein with 30 kDa molecular mass.

The deduced amino acid sequence of AlyPEEC was compared to those of other alginate lyases. The only highly conserved region (Y^{345} to Q^{353}) (Fig. 2) corresponded to a previously reported identical nine amino acid region at the C-terminus of the alginate lyases from *Klebsiella pneumoniae* (AlyA),[14] *Photobacterium* sp. (AlxM)[14] and *Vibrio halioticoli* (AlyVG1 and AlyVG2).[24] Database searches revealed that AlyPEEC showed some similarity to *Streptomyces* chitinases. In particular, some conserved regions were observed between a domain A^{32} to G^{164} of AlyPEEC and chitin binding domains of chitinases Chi01 from *Streptomyces olivaceoviridis*,[25] and

ChiA[26] and ChiB from *Streptomyces lividans*[27] (Fig. 4(A)). The degree of identity was 29% for Chi01, 31% for ChiA, and 27% for ChiB.

3. Discussion

P. elyakovii was originally isolated from a spot-wound lesions in a decaying frond of *Laminaria* and may have been the causative agent in the formation of this lesion.[18,19] The extracellular alginate lyase is considered to be a key virulence factor and integral to the catabolism of alginate by *P. elyakovii*. The alginate lyase, which has been purified from the culture supernatant of this bacterium,[19] is unusual due to its unique broad substrate specificity.[21]

In this paper, we report the cloning and sequencing of a *P. elyakovii* gene, *alyPEEC*, which encodes an alginate lyase. There is good evidence that the gene *alyPEEC* encodes the previously purified extracellular alginate lyase, because of (i) the identical sequence of 30 N-terminal amino acid residues (Fig. 2 and Ref. 21); (ii) the broad substrate specificity of the gene product;[21] and (iii) the similarity of the theoretical and experimental molecular masses (Fig. 3 and Refs. 20,21). However, interestingly, the identical 30 amino acid sequence was found in the internal region of the gene, G^{165} to V^{194}, and not at the N-terminus as expected (Fig. 2). The theoretical molecular mass (43.2 kDa) of AlyPEEC is significantly greater than the previously purified enzyme (32 kDa).[20,21] However, the molecular mass (25.4 kDa) of the domain G^{165} to N^{398} of AlyPEEC is closer to the experimental value for the purified enzyme. In fact, the results for detecting alginate lyases from cell-free extracts of clones carrying *alyPEEC* gene demonstrated that 42 and 30 kDa proteins derived from the expression of *alyPEEC* have enzymic activity (Fig. 3). The activity of the 30 kDa protein was greater than that of the 42 kDa (Fig. 3). Moreover, as strong alginate lyase activity was detected in culture supernatants of pTPB31 in the presence of IPTG (Table 1), it implies that AlyPEEC has a functional signal sequence. The most probable signal sequence was predicted by the PSORT program to be M^{10} to A^{31}. The theoretical molecular mass of the domain A^{32} to N^{398} excluding the signal sequence is 42 kDa, which corresponds to one of the alginate lyase active proteins detected by activity staining (Fig. 3). A cellular fractionation experiment of pTPB24 and pTPB31 to determine the localization of AlyPEEC expressed in *E. coli* cells suggested that AlyPEEC was likely to be present in the periplasmic space as the 30 kDa processed protein (Fig. 4). Subcloning of the domain G^{165} to N^{398} into an expression vector, pTrcHisB, confirmed that the gene product from this domain had alginate lyase activity (Fig. 5). Therefore, it is proposed that the primary transcript of *alyPEEC* is M^{10} to N^{398} with subsequent removal of the signal sequence, M^{10} to A^{31}, and an additional N-terminal region, A^{32} to G^{164}.

The function of the cleaved N-terminal domain of AlyPEEC, A^{32} to G^{164} is unknown. Homology search results showed high identity scores to three *Streptomyces* chitinases, Chi01 of *S. olivaceoviridis*,[25] ChiA[26] and ChiB[27] of *S. lividans*. As the gene product of *alyPEEC* did not have measurable chitinase activity, it is unlikely it is a bifunctional chitinase/alginate lyase. Putative primary structures of the *Streptomyces* chitinases have been reported,[27] and signal sequences, chitin binding, Fibronectin type III-like, and catalytic domains were identified. The highest identity (approximately 30%) was observed between the A^{32} to G^{164} domain of AlyPEEC and a region corresponding to the putative chitin-binding domain (Fig. 6(A)). Therefore, the A^{32} to G^{164} domain may have a similar function to the chitin-binding region. Chitin binding domain-like regions have not been observed in the 11 other alginate lyases for which the primary structure has been determined (AlgL of *Azotobacter vinelandii* AF027499; AlgL of *A. chroococcum* AJ223605;[7] AlyII of *Pseudomonas* sp. AB003330;[8] A1-I of *Sphingomonas* sp. PRF 2009330A;[9] AlyP of *Pseudomonas* sp. D38469;[10] AlgL of *Pseudomonas aeruginosa* L09724[11] and L14597;[12] AlxM of *Photobacterium* sp. X70036;[13] AlyA of *K. pneumoniae* L19657;[14] AlyA of *Pseudomonas alginovora* X83679;[15] *Turbo cornatus* polymannuronate lyase;[28] AlyL of *Pseudomonas syringae* subsp. *syringae* AF222020[16]

and an oligoalginate lyase Oal of *Sphingomonas* sp. AB011415,[17] and an epimerase AlgG of *P. aeruginosa* U27829[29]). On the basis of these results and analyses, we propose a putative domain structure for AlyPEEC (Fig. 6(B)). AlyPEEC has a signal sequence M^{10} to A^{31}, a chitin binding like-region A^{31} to G^{164}, and a mature enzyme domain G^{165} to N^{398}.

The specific activities of the gene products in LB broth containing 50% seawater were ten times higher than those in cultures without seawater (Table 1), although the reason for this is unknown. However, the extracellular alginate lyase activity produced by the host strain, *P. elyakovii*, was also stimulated by an addition of seawater or magnesium chloride and sodium chloride, which are components of seawater.[19] Therefore, these components of seawater may be concerned with folding or maintaining stability of the expressed enzyme.

Further work on the cell-bound alginate-oligosaccharide degrading enzyme of *P. elyakovii* will be needed to understand fully the catabolism of alginate by this bacterium. In the meantime, optimization of the expression of recombinant AlyPEEC will facilitate the use of the alginate lyase for the production of alginate oligosaccharides for food applications. Work is also underway to determine the detailed enzymic mechanism of AlyPEEC.

4. Experimental

Bacterial strains, plasmids and media.—*P. elyakovii* IAM 14594[18,19] was grown on AI2 agar medium containing 0.4% sodium alginate, 0.1% ammonium chloride, 0.01% yeast extract, 1.5% agar, 75% (v/v) ASW, pH 7.5 at rt.[21] *E. coli* JM109 (*rec*A1, *sup*E44, *end*A1, *hsd*R17, *gry*A96, *rel*A1, *thi*Δ (*lac-pro*AB), F′[*tra*D36, *pro*AB, *lac*I^q *lac*ZΔM15])[30] was used as the host for construction of the gene library, subcloning and expression. *E. coli* JM109 was grown on Luria–Bertani (LB) agar containing 100 μg/mL ampicillin at 37 °C. Plasmid pUC18 and pUC118[30] were used for cloning alginate lyase genes, subcloning and sequencing.

Alginate lyase expressed clones were selected using LB agar plates, containing 100 μg/mL ampicillin, 50% (v/v) ASW and 0.5% sodium alginate, pH 7.5, and, where appropriate, 100 μL of 100 mM IPTG solution spread

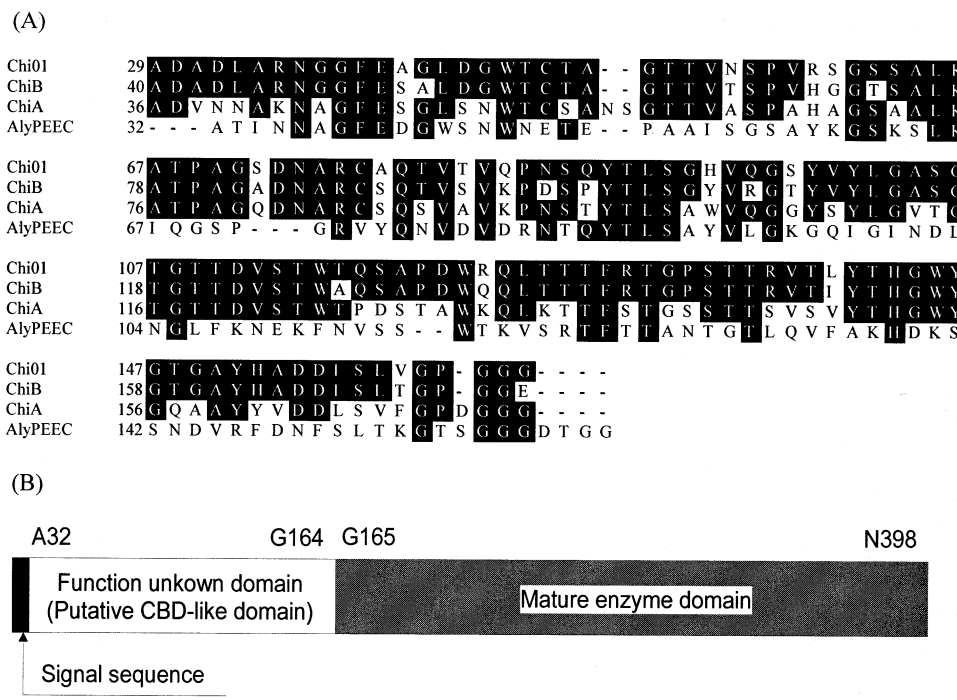

Fig. 6. Alignment of the AlyPEEC region (A^{32} to G^{165}) against chitin binding domains of *Streptomyces* chitinases (A) and a putative domain structure of AlyPEEC (B).

on the surface of each plates. Clearing zone formation around colonies was observed after incubation at 30 °C for 5 days by flooding plates with 70% (v/v) EtOH.[19]

Construction of recombinant plasmids.—Genomic DNA of *P. elyakovii* IAM 14594 was isolated using Promega Wizard™ genomic DNA extraction kit according to the manufacturers instructions. Recombinant plasmids were constructed according to Sambrook et al.[30] The genomic DNA of *P. elyakovii* was completely digested with *Hin*dIII, which produced fragments ranging from 0.7 to 7.0 kb. These fragments were purified and inserted into the *Hin*dIII site of pUC118 and transformed into *E. coli* JM109. Plasmids were purified using a Promega Wizard™plus miniprep DNA purification kit. Clones expressing alginate lyase were used as donors in subcloning experiments, and a series of subclones was established to determine the location of the alginate lyase gene. For purification of fragments, Promega Wizard™ PCR preps kits were used according to the manufacturers instructions. Then, the thermosequenase with seven deaza gtp cycle sequencing kit (Pharmacia biotech, rpn 2438) and IRD800 labeled primers (MWG Biotech-U) were used for sequencing reactions and analyzed using a DNA4000L model automated fluorescent DNA sequencer (Licor).

Sequence analysis.—Putative translation frames were identified using the Expasy translate tool on an internet site (http://www.expasy.org/). Theoretical p*I* and MW were also calculated using the Expasy Compute p*I*/MW tool. The FASTA program was used for homology searches.[31] Putative prokaryotic signal sequences were predicted using the PSORT program (http://psort.nibb.ac.jp).[32] Pairwise and multiple sequence alignments between AlyPEEC and other protein sequences were obtained using the CLUSTALW program.[33]

Nucleotide sequence accession number.—The nucleotide sequence reported in this study is listed in GenBank nucleotide sequence database under the accession number AF082561.

Assay for alginate lyase activity and detection of activity on gels.—Alginate lyase activity was assayed using the thiobarbituric acid (TBA) method.[4] The alginate lyase expressing transformant pTPB31 was cultured with LB broth or LB broth containing 50% (v/v) ASW with or without 5 mM IPTG at 30 °C. All media contained 100 μg/mL ampicillin. After 30 h incubation, the culture medium was centrifuged (8000*g* for 10 min at 4 °C). Cells were vortexed in the presence of 50 μL $CHCl_3$ and then suspended in 1.0 mL of 0.1 M Tris–HCl buffer, pH 7.5. The suspensions were kept at −20 °C overnight, and centrifuged (12,000*g* for 5 min at 4 °C), to produce cell-free extracts. The centrifuged culture medium (supernatants) and the cell-free extracts were used for enzyme assays. The reaction mixture was composed of 0.1 M Tris–HCl buffer, pH 7.5 and 0.1% sodium alginate. MM, GG and MG block were prepared by mild hydrolysis of sodium alginate,[34] and characterized by Fourier transform ^{1}H NMR; F_G 0.061 and F_M 0.939 for MM block, F_G 0.845 and F_M 0.155 for GG block, and F_G 0.398, F_M 0.602 and $F_{MG} = F_{GM}$ 0.258 for MG block. These substrates were used for determining the substrate specificity of the gene product.

The expressed alginate lyases were also detected using a combination of a substrate-containing gel overlay[21,35] after SDS–PAGE and renaturation.[36] After SDS–PAGE of cell-free extracts, enzymes were renatured by incubating for three times 30 min in 1% caseine–50 mM Tris–HCl buffer, pH 8.2, at 4 °C according to Peciña and Paneque.[36] The polyacrylamide gel was washed with 0.1 M Tris–HCl buffer, pH 7.5, then overlaid onto a gel contained 0.5% sodium alginate, 50 mM $MgCl_2$, 0.1 M Tris–HCl, pH 7.5, and 2.0% (w/v) agarose, and incubated overnight at 30 °C. The agarose gel was immersed into 10% (w/v) cetylpyridinium chloride solution, and alginate lyase activities were visualized as clear bands. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

Chitinase activity was measured using *p*-nitrophenyl-β-D-*N*,*N*′-diacetylchitobiose and *p*-nitrophenyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose[25] in 0.1 M Tris–HCl buffer, pH 7.5, at 30 °C.

Preparation of periplasmic and cytoplasmic fractions.—Periplasmic and cytoplasmic fractions were prepared according to Reverchon et al.[37] with minor modifications. Clones, pTPB24 and pTPB31, were cultured in 200

Table 3
Mismatch primers for subcloning of AlyPEEC-mature enzyme domain

Primer	Sequence and mismatch position [a]
	Ala $G^{165} \rightarrow N^{398}$
PEEC-CAT-F	5'-C T G C A G G C G A C A C T G G T A G T G G T*T C T -3'
	*Pst*I
	Thr Gly Stop $N^{398} \rightarrow G^{165}$
PEEC-CAT-R1	5'-G G T A C C T T A G*T T G*G T T C G*G*C G C G T G T -3'
	*Kpn*I

[a] Positions with mismatch in each primer were indicated by asterisk. Restriction site, which was indicated by underline, was added in each primer

mL of LB broth containing 50% ASW, 100 μg/mL ampicillin, and 5 mM IPTG for 30 h at 30 °C. The culture was centrifuged and the culture supernatant (SP fraction) and cell pellets obtained. The cell pellet was washed twice with 30 mM NaCl–10 mM Tris–HCl buffer (pH 7.3), suspended in 200 mL of ice cold 20% (w/v) sucrose–1 mM EDTA–50 mM Tris–HCl buffer (pH 7.4), and incubated for 10 min at 4 C with gentle agitation. Then the pellet was centrifuged, and resuspended in 50 mL of ice cold 5 mM $MgCl_2$ solution for 10 min at 4 °C with gentle agitation, and centrifuged. The supernatant, including periplasmic enzymes (PP fraction) and cell pellet, were collected. To disrupt the cells, the pellet was suspended in 50 mL of ice cold 10 mM Tris–HCl buffer (pH 7.3), sonicated at 50 W for five periods of 30 s. The supernatant including cytoplasmic enzymes (CP fraction) was collected after centrifugation. JM109 harboring pUC18 was also cultured in LB broth or LB broth containing 50% ASW, and SP, PP and CP fractions were prepared as described above. Alginate lyase, alkaline phosphatase and β-galactosidase activities were assayed in each fraction.[37,38]

Subcloning of alyPEEC-*mature domain and the expression.*—Since N-terminal amino acid sequences were identical to the G^{165} to V^{194} of the deduced amino acid sequence in *alyPEEC*, the region encoding G^{165} to N^{398} was amplified from the insert of pTPB31 using *Pwo* polymerase (Boehringer Mannheim) and two mismatch primers (PEEC–CAT–F and PEEC–CAT–R1, Table 3). An amplified fragment of approximately 700bp was gel-purified, digested with *Pst*I and *Kpn*I, and inserted between *Pst*I and *Kpn*I sites of the pTrcHisB expression vector (Invitrogen). Top10 *E. coli* cells were transformed with the recombinant plasmid and screened for alginate lyase positive clones.

Acknowledgements

We are grateful to Mrs M.A. Whittaker, University of Central Lancashire, for technical assistance, Mr Bill Edward, University of Wales, Cardiff, for DNA sequencing, Mr H. Tanaka, University of Hokkaido, for protein sequences of expressed proteins, and Mr P. Warren, The Manchester Metropolitan University, for NMR analysis of alginate substrates. This work was supported by Grants-in-Aid for Scientific Research from Ministry of Education, Science and Culture of Japan (no. 11760129).

References

1. Gacesa, P. *Carbohyd. Polym.* **1988**, *8*, 161–182.
2. Gacesa, P. *Int. J. Biochem.* **1992**, *24*, 545–552.
3. Sutherland, I. W. *FEMS Microbiol. Rev.* **1995**, *16*, 323–347.
4. Preiss, J.; Ashwell, G. *J. Biol. Chem.* **1962**, *237*, 309–316.
5. Williams, A. K.; Eagon, R. G. *Can. J. Microbiol.* **1962**, *8*, 649–654.
6. Wong, T. Y.; Preston, L. A.; Schiller, N. L. *Annu. Rev. Microbiol.* **2000**, *54*, 289–340.
7. Peciña, A.; Pascual, A.; Paneque, A. *J. Bacteriol.* **1999**, *181*, 1409–1414.
8. Kraiwattanapong, J.; Ooi, T.; Kinoshita, S. *Biosci. Biotech. Biochem.* **1997**, *61*, 1853–1857.
9. Yonemoto, Y.; Tanaka, H.; Hisano, T.; Sakaguchi, K.; Abe, S.; Yamashita, T.; Kimura, A.; Murata, K. *J. Ferment. Bioeng.* **1993**, *75*, 336–342.

10. Maki, H.; Mori, A.; Fujiyama, K.; Kinoshita, S.; Yoshida, T. *J. Gen. Microbiol.* **1993**, *139*, 987–993.
11. Schiller, N. L.; Monday, S. R.; Boyd, C. M.; Keen, N. T.; Ohman, D. E. *J. Bacteriol.* **1993**, *175*, 4780–4789.
12. Boyd, A.; Ghosh, M.; May, T. B.; Shinabarger, D.; Keogh, R.; Chakrabarty, A. M. *Gene* **1993**, *131*, 1–8.
13. Malissard, M.; Duez, C.; Guinand, M.; Vacheron, M. J.; Michel, G.; Marty, N.; Joris, B.; Thamm, I.; Ghuysen, J. M. *FEMS Microbiol. Lett.* **1993**, *110*, 101–106.
14. Baron, A. J.; Wong, T. Y.; Hicks, S. J.; Gacesa, P.; Willcock, D.; McPherson, M. J. *Gene* **1994**, *143*, 61–66.
15. Chavagnat, F.; Duez, C.; Guinand, M.; Potin, P.; Barbeyron, T.; Henrissat, B.; Wallachand, J.; Ghuysen, J.-M. *Biochem. J.* **1996**, *319*, 575–583.
16. Preston, L. A.; Wong, T. Y.; Bender, C. L.; Schiller, N. L. *J. Bacteriol.* **2000**, *182*, 6268–6271.
17. Hashimoto, W.; Miyake, O.; Momma, K.; Kawai, S.; Murata, K. *J. Bacteriol.* **2000**, *182*, 4572–4577.
18. Sawabe, T.; Tanaka, R.; Iqbal, M. M.; Tajima, K.; Ezura, Y.; Ivanova, E. P.; Christen, R. *Int. J. Sys. Evol. Microbiol.* **2000**, *50*, 265–271.
19. Sawabe, T.; Ezura, Y.; Kimura, T. *Nippon Suisan Gakkaishi* **1992**, *58*, 141–145.
20. Sawabe, T.; Ezura, Y.; Kimura, T. *Nippon Suisan Gakkaishi* **1992**, *58*, 521–527.
21. Sawabe, T.; Ohtsuka, M.; Ezura, Y. *Carbohyd. Res.* **1997**, *304*, 69–76.
22. Sawabe, T.; Sawada, C.; Suzuki, E.; Ezura, Y. *Fisheries Sci.* **1998**, *64*, 320–324.
23. Sato, R.; Sawabe, T.; Kishimura, H.; Hayashi, K.; Saeki, H. *J. Agric. Food Chem.* **2000**, *48*, 17–21.
24. Sugimura, I.; Sawabe, T.; Ezura, Y. *Mar. Biothechnol.* **2000**, *2*, 65–73.
25. Blaak, H.; Schnellmann, J.; Walter, S.; Henrissat, B.; Schrempf, H. *Eur. J. Biochem.* **1993**, *214*, 659–669.
26. Miyashita, K.; Fujii, T. *Biosci. Biotech. Biochem.* **1993**, *57*, 1691–1698.
27. Miyashita, K.; Fujii, T.; Watanabe, A.; Ueno, H. *J. Ferment. Bioeng.* **1997**, *83*, 26–31.
28. Muramatsu, T.; Komori, K.; Sakurai, N.; Yamada, K.; Awasaki, Y.; Fukuba, K.; Oda, T. *J. Protein Chem.* **1996**, *15*, 709–719.
29. Franklin, M. J.; Chitnis, C. E.; Gacesa, P.; Sonesson, A.; White, D. C.; Ohman, D. E. *J. Bacteriol.* **1994**, *176*, 1821–1830.
30. Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning. A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, 1989.
31. Pearson, W. P. *Methods Enzymol.* **1990**, *183*, 63–98.
32. Nakai, K.; Kanehisa, M. *Proteins: Structure, Function and Genetics* **1991**, *11*, 95–110.
33. Thompson, J. D.; Higgins, D. G.; Gibson, T. J. *Nucleic Acids Res.* **1994**, *22*, 4673–4680.
34. Haug, A.; Larsen, B.; Smidsrød, O. *Acta Chem. Scand.* **1967**, *21*, 691–704.
35. Caswell, C. R.; Gacesa, P.; Weightman, A. J. *Int. J. Biol. Macromol.* **1986**, *8*, 337–341.
36. Peciña, A.; Paneque, A. *Anal. Biochem.* **1994**, *217*, 124–127.
37. Reverchon, S.; Hugouvieux-Cotte-Pattat, N.; Robert-Baudouy, J. *Gene* **1985**, *35*, 121–130.
38. Gacesa, P.; Goldberg, J. B. *J. Gen. Microbiol.* **1992**, *138*, 1665–1670.