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A novel alginate lyase with high activity on acetylated alginate of *Pseudomonas aeruginosa* FRD1 from *Pseudomonas* sp. QD03

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Summary

To exploit alginate lyase which could degrade bacterial alginates, degenerate PCR and long range-inverse PCR (LR-IPCR) were used to isolate alginate lyase genes from soil bacteria. Gene *algL*, an alginate lyase-encoding gene from *Pseudomonas* sp. QD03 was cloned, and it was composed of a 1122 bp open reading frame (ORF) encoding 373 amino acid residues with the calculated molecular mass of 42.2 kDa. The deduced protein had a potential N-terminal signal peptide of 20 amino acid residues that was consistent with its proposed periplasmic location. Gene *algL* was expressed in pET24a (+)/*E. coli* BL21 (DE3) system. The recombinant AlgL was purified to electrophoretic homogeneity using affinity chromatography. The molecular weight of AlgL was estimated to be 42.8 kDa by SDS-PAGE. AlgL exhibited maximal activity at pH 7.5 and 37 °C. Na^+, K^+, Ca^{2+} and Ba^{2+} significantly enhanced the activity of AlgL. AlgL could degrade alginate and mannuronate blocks, but hardly degrade guluronate blocks. In particular, AlgL could degrade acetylated alginate of *Pseudomonas aeruginosa* FRD1 (approximately 0.54 mol of *O*-acetyl group per mol of alginate). It might be possible to use alginate lyase AlgL as an adjuvant therapeutic medicine for the treatment of disease associated with *P. aeruginosa* infection.

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) (M blocks) and poly (α-L-gulosyluronic acid) (G blocks)] or heteropolymeric, i.e. containing random blocks (MG blocks) (Gacesa 1992; Sutherland 1995). Alginates are synthesized as cell wall components by brown seaweeds and as exopolysaccharides by some bacteria belonging to the genera *Azotobacter* and *Pseudomonas* (Govan *et al.* 1981; Cote *et al.* 1988; Smidsrød *et al.* 1996). In contrast to alginate synthesized by the algae, these bacteria produce polysaccharides often substituted with *O*-acetyl groups on the 2 and/or 3 positions of D-mannuronate (Skjåk-Bræk *et al.* 1985). In addition, alginate produced by *Pseudomonas* contains no poly(G) region (Jost *et al.* 2001). The susceptibility of alginates to degradation is determined by both the block structure and degree of *O*-acetylation within the macromolecule (Wong *et al.* 2000).

The research of *Pseudomonas* strains is becoming more important because of their ubiquitous environmental distribution and wide association with human infections, such as cystic fibrosis lung infections (Peter 1998). Most *Pseudomonas* strains produce large amount of extracellular alginates (Fett *et al.* 1992). Because of the high molecular mass of bacterial alginate and the negative charge, the polysaccharide is highly hydrated and viscous. Alginates represent major components of the extracellular polymeric substances (EPS) of mucoid *P. aeruginosa* and have been implicated in the development as well as the maintenance of the mechanical stability of biofilm formed by *P. aeruginosa* on living and abiotic surface (Davies 1999; Jost *et al.* 2001). Formation of these sessile communities and their tolerance to antimicrobial agents and host defences are at the root of many persistent and chronic bacterial infections (Costerton *et al.* 1999).

Alginate lyase cleaves the glycosidic linkage of alginate through a β-elimination reaction and gives rise to unsaturated oligouronic acids having 4-deoxy-L-erythro-hex-4-enopyranosyluronic acid at the nonreducing end (Wong *et al.* 2000). Based on their primary structures, alginate lyases are grouped into three families, PL-5, -7, and -14 (Henrissat *et al.* 2005). Most of the family PL-5 and -7 alginate lyases specifically depolymerize poly(M) and poly(G), respectively, although family PL-14 contains enzymes specific for poly(M) or poly(G) (Yamasaki *et al.* 2004). Alginate lyase removes the exopolysaccharide from the surface of mucoid pseudomonal cells *in vivo* and *in vitro* (Bayer *et al.* 1992) and inhibits adherence of the mucoid strain of *P. aeruginosa* (Mai *et al.* 1993b). Algi-

nate lyase promoted diffusion of aminoglycosides through the extracellular polysaccharide of mucoid *P. aeruginosa* (Richard *et al.* 1998). It rendered *P. aeruginosa* cells more susceptible to amikacin-induced post-antibiotic leukocidal enhancement *in vitro* (Bayer *et al.* 1991) and reversed suppression of lymphocyte and neutrophil functions by *P. aeruginosa* mucoid exopolysaccharide (alginate) (Mai *et al.* 1993a). Thereby, the lyase might be used as an adjuvant therapeutic agent for the treatment of infection by mucoid strains of *P. aeruginosa*. Since the original description of alginate lyases more than 50 years ago, many examples have been isolated (Wong et al. 2000). However, most of them could not degrade bacteria *O*-acetylated alginate, and some were able to attack bonds where one of the residues was acetylated but might not be able to attack bonds linking two acetylated residues (Ertesvåg *et al.* 1998). So there are only a few lyases effective on acetylated alginate, such as the alginate lyase of *Azotobacter vinlandii* (Ertesvåg *et al.* 1998, GenBank AF037600), Al_XM_B of *Photobacterium* (Maslissard et al. 1993, GenBank X70036), PA3547 of *P. aeruginosa* PAO1 (Yamasaki *et al.* 2004, GenBank NC_002516) and A1-I and A1-III of *Sphingomonas* sp. A1 (Yonemoto et al. 1992; Hisano *et al.* 1993, Protein Research Foundation 2009330A; Protein Data Bank 1QAZ). All of those acetylated alginate lyases could degrade M blocks, but not all of those M-specific lyases showed activity against acetylated bacterial alginate (Wong *et al.* 2000).

In this paper, degenerate primers were designed corresponding to the conserved regions of M-specific lyases, a novel alginate lyase gene *algL* was cloned from soil bacterium *Pseudomonas* sp. QD03 by degenerate PCR and LR-IPCR. The enzyme efficiently degraded acetylated alginate of *P. aeruginosa* FRD1, and this value almost equalled its activity against M blocks.

Materials and methods

Bacterial strains and cultural conditions, oligonucleotides

Escherichia coli DH5α (Gibco BRL) and lysogenic *E. coli* BL21 (DE3) (Novagen) were used as hosts for recombinant plasmids. *E. coli* cells were grown at 37 °C in Luria–Bertani (LB) broth (Sambrook & Russell 2001) or on LB agar supplemented with ampicillin (50 μg/ml) or kanamycin (30 μg/ml) when relevant. *Pseudomonas* sp. QD03, which was isolated from soil, was grown at 25 °C in LB broth. Mucoid strain *P. aeruginosa* FRD1 (a gift from Prof Ohman) was originally obtained from the sputum of patients with cystic fibrosis (Ohman & Chakrabarty 1981). *P. aeruginosa* FRD1 was grown at 37 °C in LB broth supplemented with Irgasan (25 μg/ml). Plasmid pBluescript□KS (+) (Stratagene) was used as cloning vector. Plasmid pET-24a (+), which was used for expression of recombinant protein, was from Novagen. Oligonucleotides were synthesized by Bioasia Company (Shanghai, P.R. China) on a Perkin–Elmer ABI synthesizer (Perkin–Elmer).

Isolation and identification of strain Pseudomonas *sp. QD03*

Strain *Pseudomonas* sp. QD03 was isolated from soil collected from Qingdao, China. One gram of soil sample suspended in 10 ml of distilled water, filtered through a 0.45 μm-pore size membrane. Filtrate was diluted and laid on the solid LB plate, and then cultured at 25 °C for 20 h. Colonies were randomly picked up from the plates and cultured in 96-well microtitre plates. Degenerate primers D1 (5′-WBBAACAACCACTCVTACTGG-3′) and D2 (5′-BGHACARSAGGGTTCCAGCCA-3′) designed corresponding to conserved regions NNHSYW and WLEPXCXLY (X is the variable residue) of M-specific lyases were used to screen the positive strain carrying the alginate lyase gene. Clones cultured in the above 96-well microtitre plates were used as the template of the degenerate PCR. Degenerate PCR was carried out for 25 cycles, each cycle with 1 min denaturation at 94 °C, 30 s annealing at 62 °C, 50 s extension at 72 °C. The final elongation step at 72 °C was for 10 min. According to the PCR product and sequence analysis, positive clones were obtained. Morphological, physiological, biochemical characteristics and 16S rRNA analyses were used to identify the isolated strain (Oliver 1982; Polz & Cavanaugh 1998).

Cloning of algL gene from Pseudomonas *sp. QD03 and nucleotide sequence analysis*

Standard recombinant DNA procedures were performed according to protocols described by Sambrook & Russell (2001). All the enzymes used in this study were obtained from TaKaRa (Dalian, P. R. China) unless stated otherwise. To obtain the full *algL* gene, the specific primers, S1 (5′-GGGCCGTGAAGGAATACAAG-3′) and S2 (5′-GAGAACTTCAGGCGGACATA-3′) of long range-inverse PCR (LR-IPCR) were designed according to the degenerate PCR product from positive clone, *Pseudomonas* sp. QD03, and LR-IPCR was carried out as described by Benkel & Fong (1996). Briefly, genomic DNA of *Pseudomonas* sp. QD03 was partially digested with *Sau*3AI, and separated on a 0.7% agarose gel. DNA fragments of approximately 2.0–6.0 kb were recovered from agarose gel and religated. The ligation mixture was used as the template of LR-IPCR. Then, LR-IPCR fragment was sequenced. Degenerate PCR fragment and LR-IPCR fragment were joined together according to the overlapping of them.

The putative translation frames were identified by DNATools program, and theoretical molecular mass and isoelectric point were calculated using Compute pI/Mw tool (http://us.expasy.org/tools/pi_tool.html) and ProtParam tool (http://us.expasy.org/tools/protparam.html). The putative signal peptide was predicted using the PSORT program (http://psort.ims.u-tokyo.ac.jp) and TargetP program (http://www.cbs.dtu.dk/services/TargetP). The FASTA program was used for homology searches. Pairwise and multiple sequence

alignments between AlgL and other known alginate lyase sequences were obtained using the CLUSTALW program.

Expression and purification of AlgL

To express the gene *algL*, the primers Lupper and Llower were designed. Lupper (5′-GGAATTCCA-TATGAGGTTATCTATGCAGAAG-3′) contained a *Nde*I site (underlined) and Llower (5′-CGCGGATCC ATGGAACCTTTGTTGCCTTTTT-3′) contained a *Bam*HI site (underlined). Gene *algL* was amplified through PCR using *pfu* DNA polymerase (Sangon, Shanghai, P.R. China), with genomic DNA of *Pseudomonas* sp. QD03 as the template. After gel purification, the PCR fragment was digested with *Nde*I and *Bam*HI, and ligated into a similarly digested pET-24a (+) expression plasmid, yielding pET24-algL, which expressed a recombinant protein containing 6xHis-Tags at C-terminus.

Escherichia coli BL21 cells holding plasmid pET24-algL were grown at 37 °C in 1 litre of LB containing kanamycin (30 μg/ml) until the optical density at 600 nm was about 0.5. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.1 mM and the culture was incubated at 18 °C for an additional 16 h. Cells were harvested by centrifugation ($5000 \times g$ for 15 min), and suspended in 150 ml of Osmotic Shock Solution I (20 mM Tris–HCl, pH 8.0, 2.5 mM EDTA, 20% sucrose). After being incubated on ice for 10 min, cells were harvested by centrifugation ($5000 \times g$ for 15 min), resuspended cells in 150 ml of Osmotic Shock Solution II (20 mM Tris, pH 8.0, 2.5 mM EDTA), then incubated on ice for 10 min. The solution was centrifuged and the supernatant (500 mM NaCl supplied) was mixed with 10 ml of Ni–NTA agarose (Invitrogen Inc.) followed by gently shaking on ice for 1 h. The mixture was loaded onto an empty Probond Resin column (Invitrogen Inc.) and the unbound proteins were washed away with 2×8 ml aliquots of 100 mM phosphate buffer (PB, pH 7.5, containing 500 mM NaCl). Bound proteins were eluted out fractionally with 10.0 ml of 100 mM PB (pH 7.5, containing 500 mM NaCl) supplemented with 100, 150, or 250 mM imidazole, respectively. Hexa-His tagged alginate lyases eluted by 250 mM imidazole-containing buffer were pooled and dialysed against 100 mM PB (pH 7.5), then stored at −20 °C. The protein concentration was estimated by the Lowry method, using bovine serum albumin (BSA) as a standard. SDS-PAGE was performed using a 12% gel at 16 °C with a PhastSystem™ (Pharmacia Biotech), and the gels were stained with Coomassie Brilliant Blue.

Isolation of bacterial alginate and measurement of acetyl content of the bacterial alginate

The acetylated alginate was isolated from the mucoid strain *P. aeruginosa* FRD1 as described previously (Thomassen *et al.* 1979). Briefly, 250 ml of cultures were mixed with 250 ml of saline to reduce viscosity, and the cells were removed by centrifugation ($25000 \times g$ for 15 min). The culture supernatant was mixed with 250 ml of 2% cetylpyridinium chloride (Sigma), and the precipitation alginate was collected by centrifugation ($25000 \times g$ for 15 min). The pellet was dissolved in 25 ml of 1 M NaCl, precipitated again with 25 ml of cold (−20 °C) isopropanol, and dissolved in substrate solution (1 mg/ml in 100 mM phosphate buffer, pH 7.5).

The acetyl content of the bacterial alginate was determined by a photometric assay according to Hestrin *et al.* (1949). Briefly, 500 μl of alginate solution was incubated with 500 μl of alkaline hydroxylamine (0.35 M NH_2OH, 0.75 M NaOH) for 10 min at 25 °C. The reaction mixture was acidified with 500 μl of 1.0 M perchloric acid, and 500 μl of 70 mM ferric perchlorate in 0.5 M perchloric acid was added. The concentration of acetyl groups was determined spectrophotometrically at 500 nm on the basis of a standard curve with ethyl acetate as the substrate.

Measurement of alginate lyase activity

The substrates were acetyl-group-free alginate (60% mannuronate, Sigma), the acetylated alginate (approximately 0.54 mol of *O*-acetyl group per mol of alginate) was isolated from the mucoid strain *P. aeruginosa* FRD1, M blocks (95% mannuronate) and G blocks (91% guluronate) (provided by Dr Zhao Yang) were prepared from brown seaweed alginate as described by Haug *et al.* (1974). The concentration of alginate in culture supernatants was determined by the carbazole method of Knutson *et al.* (1968). The activities of AlyVI of *Vibrio* sp. QY101 (Han *et al.* 2004) and alginate lyase AlgL of *Pseudomonas* sp. QDA (Han *et al.*, unpublished data) on acetylated alginate of *P. aeruginosa* FRD1 were analysed in parallel asssays. AlyVI was prepared as described previously (Han *et al.* 2004). The gene *algL* of *Pseudomonas* sp. QDA was expressed in pET24a (+)/*E. coli* BL21 (DE3) system, and the recombinant alginate lyase was purified to electrophoretic homogeneity using affinity chromatography (Han *et al.* unpublished data).

Reaction mixture (500 μl) containing 0.1% substrate, 20 mM PB (pH 7.5) and an appropriate amount of enzyme at 37 °C was incubated for 30 min, and the absorbance at 235 nm was read continuously. One unit (U) of alginate lyase is defined as an increase of 0.1 in absorbance at 235 nm per minute.

Fluorophore-assisted carbohydrate electrophoresis (FACE)

Products derived from poly(G) and poly(M) through the reactions of AlgL were heated at 100 °C for 10 min to terminate the reaction. After centrifugation the supernatant was mixed with equal volume of 5% ANDS (7-amino-1,3-naphthalenedisulphonic acid monohydrate) dissolved in 15% acetic acid, incubated at room

temperature for 1 h, then mixed with 0.5 volume of 1 M sodium cyanoborohydride and incubated at 45 °C for 12 h. The procedures employed for vertical gel electrophoresis were essentially the same as described previously (Shimokawa *et al.* 1996). Mini gels of 10 × 10.5 × 0.1 cm with 10 wells of 1.1 × 0.35 cm were used. Each fluorophore-labelled mixture was mixed with an equal volume of 50% (w/v) sucrose loading buffer and used as samples. The gel was loaded with 10 μl of sample in each well and subjected to electrophoresis at 250 V at 6–8 °C for 4 h, achieved by cooling in a circulating bath. The gels were visualized in ultraviolet light.

Polyacrylamide gels electrophoresis (PAGE) of carbohydrate

Product derived from bacterial alginate through the reaction of AlgL was heated at 100 °C for 10 min to terminate the reaction. The procedures employed for vertical gel electrophoresis were essentially the same as described previously (Min *et al.* 1986). The gels were stained with Alcian blue (0.1% in 1% acetic acid).

Results and discussion

Multiple alignment protein sequences of M-specific lyases of *Azotobacter*, *Pseudomonas* and *Halomona marina* showed that there were markedly conserved regions, especially notable was the highly conserved 6-amino-acid hydrophilic sequence "NNHSYW" in the centre of the protein sequences. There was also a semiconserved 9-amino-acid hydrophobic sequence "WLEPXCXLY" (X is the variable residue) in the C terminus of the lyases. The site-directed mutagenesis study of *algL* gene of *P. syringae pv. Syringae* had identified that the hydrophilic sequence NNHSYW (residues 202–207) which was essential for catalytic activity, might also serve as alginate-binding motif (Loria *et al.* 2000). In conjunction with alginate produced by *Pseudomonas* which contained no poly(G) region and most of those acetylated alginate lyases to date could degrade M blocks, degenerate PCR based on the two conserved regions of M-specific lyases were used to exploit novel lyases with activity against highly acetylated bacterial alginate from environmental microbial community.

Isolation and identification of strain Pseudomonas *sp. QD03*

As the result of degenerate PCR screening, one positive clone carrying a 400 bp PCR product was obtained, and then the PCR fragments were sequenced. FASTA analysis revealed that the 400 bp fragment had homology to published sequences of alginate lyase. According to the morphological, physiological, biochemical characteristics and 16S rRNA analyses (data not shown), the positive clone was identified as the Gram-negative bacterium *Pseudomonas* and named as *Pseudomonas* sp. QD03.

Cloning and sequence analysis of gene algL encoding alginate lyase

A 5.1 kb DNA fragment from the genomic DNA of *Pseudomonas* sp. QD03 was obtained by LR-IPCR. Sequence analysis showed overlapping of previously sequenced 400 bp PCR fragment with regions in the 5.1 kb DNA, and the full alginate lyase-encoding gene *algL* was cloned from *Pseudomonas* sp. QD03. The nucleotide sequence and deduced amino acid sequence of *algL* (GenBank Accession No. AY380832) are shown in Figure 1. It was composed of a 1122-bp open reading frame (ORF), which started with an ATG codon at position 70 and terminated with a TGA codon at position 1189. The possible ribosome-binding site (RBS) and promoter and terminator were not found in the nucleotide sequence. This might be due to this alginate lyase being located in the alginate biosynthetic operon, as the regulation of all the genes in the biosynthetic operon is complex and involves specific gene products and those that act more globally (Gacesa 1998). The predicted product, AlgL, of 373 amino acid residues M^1–S^{373} had a theoretical molecular mass of 42.2 kDa and a predicted isoelectric point of 8.79. The region M^1–A^{20} was estimated as putative signal peptide using PSORT program and TargetP program. There were a highly conserved hydrophilic NNHSYW motif in the center of the protein and a semi-conserved hydrophobic WLEPFCTLY region in the C-terminus (Figure 1). FASTA searches revealed that the deduced amino acid sequence of AlgL from *Pseudomonas* sp. QD03 showed 93% identity to AlgL of *P. fluorescens* (GenBank AF527790), 67% identity to alginate lyase of *Pseudomonas* sp. QDA, 59% identity to PA3547 of *P. aeruginosa* PAO1, 56% identity to alginate lyase of *Azotobacter vinlandii*, 24% identity to A1-III lyases of *Sphingomonas* sp. A1. AlgL of *P. fluorescens* had very recently been deposited in the GenBank database, but there was no available report on characterization of AlgL of *P. fluorescens*. Additionally, most residues of AlgL were hydrophilic and the signal peptide was A-and L-rich (Figure 1), suggesting that it was a periplasm-localized enzyme similar to that of *P. aeruginosa* (Boyd *et al.* 1993; Schiller *et al.* 1993).

Expression and characterization of recombinant AlgL

Escherichia coli BL21 (DE3) cells containing plasmid pET24-algL were used to express *Pseudomonas* sp. QD03 *algL* with its own putative M^1–A^{20} signal peptide and the His-tag fused at the C-terminus. Recombinant AlgL was purified by chromatography on a Ni^{2+} resin column from the Osmotic Shock Solution II (specific activity 9.8 U/mg in 100 mM PB, pH 7.5). Approximate 10.3 mg recombinant enzyme with the specific activity of 188.5 U/mg in 100 mM PB (pH 7.5) was obtained from per litre bacterial culture fluid. The molecular mass of the

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GGTCGAAGCGAAAATTTGCACACGCAACGTATTCCCAAGCGGCGGTCAGAAGACTGCCTC   60
ACTCGGGCAATGAGGTTATCTATGCAGAAGTTACTGATTCCATCGTTACCGGGCCTGGCG  120
         M  R  L  S  M  Q  K  L  L  I  P  S  L  P  G  L  A   17
ATCTTCGCCGGCGCGGCCAGCGCCGCCGCGCCACTGCGTCCACCCCAGGGCTACTTCGCA  180
 I  F  A  G  A  A  S  A  A  A  P  L  R  P  P  Q  G  Y  F  A   37
CCGATTGAAGCGTTCAAGACCGGCGATTTCAAGAATGACTGCGATGCCATGCCCACGCCG  240
 P  I  E  A  F  K  T  G  D  F  K  N  D  C  D  A  M  P  T  P   57
TACACCGGCCCGCTGCAATTTCGCAGCAAGTACGAAGGCTCCGACAAGGCCCGCTCCACG  300
 Y  T  G  P  L  Q  F  R  S  K  Y  E  G  S  D  K  A  R  S  T   77
CTGAATGTGCAGTCGGAAAAAGCCTTTCGCGACAGCACCGCCGACATCACCAAGCTGGAA  360
 L  N  V  Q  S  E  K  A  F  R  D  S  T  A  D  I  T  K  L  E   97
AAAGACACCAGCAAGCGCGTGATGCAGTTCATGCGCGACGGTCGTCCGGAGCAGTTGGAA  420
 K  D  T  S  K  R  V  M  Q  F  M  R  D  G  R  P  E  Q  L  E  117
TGCACGCTCAACTGGTTGACCAGCTGGGCCAAGGCTGACGCGTTGATGTCCAAGGACTTC  480
 C  T  L  N  W  L  T  S  W  A  K  A  D  A  L  M  S  K  D  F  137
AACCACACCGGCAAGTCCATGCGCAAATGGGCACTGGGCAGCATGGCCTCGGCCTATGTC  540
 N  H  T  G  K  S  M  R  K  W  A  L  G  S  M  A  S  A  Y  V  157
CGCCTGAAGTTCTCCGACTCGCATCCGCTGGCCAACCACCAGCAGGAATCGCAACTGATC  600
 R  L  K  F  S  D  S  H  P  L  A  N  H  Q  Q  E  S  Q  L  I  177
GAAGCCTGGTTCAACAAGCTGGCGGACCAGGTGGTCAGCGACTGGGACAACCTGCCGCTG  660
 E  A  W  F  N  K  L  A  D  Q  V  V  S  D  W  D  N  L  P  L  197
GAAAAAACCAACAACCACTCCTACTGGGCCGCCTGGTCGGTGATGGCAACCTCCATCGCC  720
 E  K  T  N  N  H  S  Y  W  A  A  W  S  V  M  A  T  S  I  A  217
ACCAACCGTCGCGACCTGTTTGATTGGGCCGTGAAGGAATACAAGGTCGGCGTCAACCAG  780
 T  N  R  R  D  L  F  D  W  A  V  K  E  Y  K  V  G  V  N  Q  237
GTCGATGACCAGGGCTTTTTGCCCAACGAGTTGAAGCGTCAGCAACGCGCCCTGTCGTAC  840
 V  D  D  Q  G  F  L  P  N  E  L  K  R  Q  Q  R  A  L  S  Y  257
CACAACTACGCGCTGCCGCCGCTGTCGATGATCGCCAGCTTTGCCCTGGTCAACGGGGTT  900
 H  N  Y  A  L  P  P  L  S  M  I  A  S  F  A  L  V  N  G  V  277
GACCTGCGCCAGGAAAACAACGGCGCGCTCAAGCGCCTGGGCGACAAGGTGCTGGCCGGG  960
 D  L  R  Q  E  N  N  G  A  L  K  R  L  G  D  K  V  L  A  G  297
GTCAAGGATCCGGAGATCTTCGAACAGAAGAACGGCAAGGAACAGGACATGAAGGATCTC 1020
 V  K  D  P  E  I  F  E  Q  K  N  G  K  E  Q  D  M  K  D  L  317
AAGGAAGACATGAAATTCGCCTGGCTCGAACCCTTCTGCACCCTCTACACCTGCGCGCCG 1080
 K  E  D  M  K  F  A  W  L  E  P  F  C  T  L  Y  T  C  A  P  337
GATGTACTCGAACGCAAGCACGGGATGCAGCCGTTCAAGACCTTCCGCCTCGGCGGCGAC 1140
 D  V  L  E  R  K  H  G  M  Q  P  F  K  T  F  R  L  G  G  D  357
CTGACCAAGGTCTACGACCCCGCACACGAAAAAGGCAACAAAGGTTCCTGAGCGAACTCG 1200
 L  T  K  V  Y  D  P  A  H  E  K  G  N  K  G  S  *           373
GTAAAAATGTGGGA                                               1214
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Figure 1. Nucleotide and deduced amino acid sequences of the novel *algL* gene from *Pseudomonas* sp. QD03. The stop codon of the ORF is indicated by the asterisk, and the possible singal peptide is indicated by a dotted underline. Shaded regions represented the conserved sequences (NNHSYW and WLEPFCTLY).

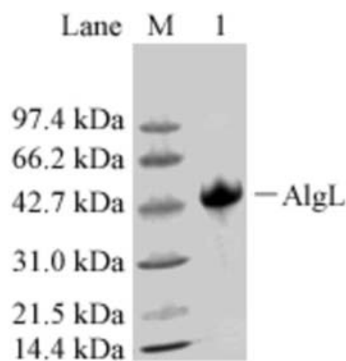

Figure 2. SDS-PAGE assay of purified AlgL. Lane M, protein markers (phosphoryfase B, 94.7 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibtor, 21.5 kDa; lysozyme, 14.4 kDa) with the size of each band indicated; Lane 1, the purified AlgL.

recombinant AlgL was determined to be 42.8 kDa by SDS-PAGE (Figure 2), which was identical to the theoretic molecular mass of the amino acid sequence of AlgL. The N-terminus of purified AlgL was sequenced, and the first five residues (GAASA) confirmed that the protein was cleaved between A^{20} and G^{21} (Data not shown).

The lyase AlgL was most active at pH 7.5 and 37 °C, respectively (Figure 3a and b). Seventy percent of the activity was lost when AlgL was incubated at 60 °C for 60 min in 100 mM PB (pH 7.5) (Figure 3c).

The activity of lyase AlgL was tested in the presence or absence of various chemical reagents (Table 1). Fe^{3+} and SDS (1 mM) were potent inhibitors of the lyase activity. Zn^{2+}, Ni^{2+} and Cu^{2+} (1 mM) partly inhibited the lyase activity. Na^{+} and K^{+} significantly enhanced the activity of AlgL. The lyase did not require $BaCl_2$ or $CaCl_2$ for activity; however, the addition of Ba^{2+} or Ca^{2+} enhanced lyase activity by 318% or 147%. EDTA had no effect on activity of the lyase. In addition, studies with an M-lyase from *P. aeruginosa* to reduce sputum viscosity *in vitro* were disappointing because Ca^{2+} and Zn^{2+} levels in most CF sputa inhibited lyase enzyme activity (Mrsny *et al.* 1994). However, Ca^{2+} observably enhanced the activity of AlgL by 147%. Zn^{2+} inhibited the activity of AlgL by 21% in the levels of 1 mM which was about 10 times higher than that of in human sputa (Mrsny *et al.* 1994), whereas Zn^{2+} hardly inhibited the lyase activity in level of human sputa (data not shown).

The substrate-specificity of AlgL was characterized by both the qualitative and quantitative assays for catalytic activity. AlgL showed lyase activities of 188.5 U/mg against sodium alginate, 158.3 U/mg against acetylated alginate of *P. aeruginosa* FRD1 and 197.7 U/mg against M blocks, respectively, whereas AlgL showed a lyase activity of only 2.3 U/mg against G blocks. AlgL of *Pseudomonas* sp. QD03 degraded M blocks and generated more polymannuronate oligomers with degree of polymerization (dp) of 3–5 (Figure 4b), whereas it did not degrade G blocks (Figure 4a). Furthermore, as shown in Figure 5, AlgL could degrade acetylated alginate from *P. aeruginosa* FRD1 (approximately 0.54 mol of *O*-acetyl group per mol of alginate). However, AlyVI from *Vibrio* sp. QY101 (Han *et al.* 2004) which preferred polyguluronate blocks as substrate showed no activity against acetylated alginate of *P. aeruginosa* FRD1. Alginate lyase of *Pseudomonas* sp. QDA (Han *et al.*, unpublished data) preferred polymannuronate blocks as substrate and had activity of 26.2 U/mg on alginate isolated from *P. aeruginosa* FRD1 in the study (Data not shown). In addition, M-specific lyase AlgL of *P. aeruginosa* showed nearly no activity against alginate with 10% acetylated alginate (Linker & Evans 1984), while a previously characterized *A. vinelandii* enzyme still had activity on 37% acetylated alginate (Kennedy *et al.* 1992). These results indicated that AlgL of *Pseudomonas* sp. QD03 showed high activity against 54% acetylated alginate, and this value almost equalled the activity against M blocks. It might be possible to use AlgL as an adjuvant therapeutic medicine for the treatment of disease associated with *P. aeruginosa* infection.

The PAGE method has been successfully applied in analysing different kinds of carbohydrates. It was particularly suitable for polysaccharides and high-molecular-weight or highly sulphated oligosaccharides. However, the major limitations of the PAGE method resulted from the failure of neutral or weakly charged molecules to migrate well under electrophoresis and the

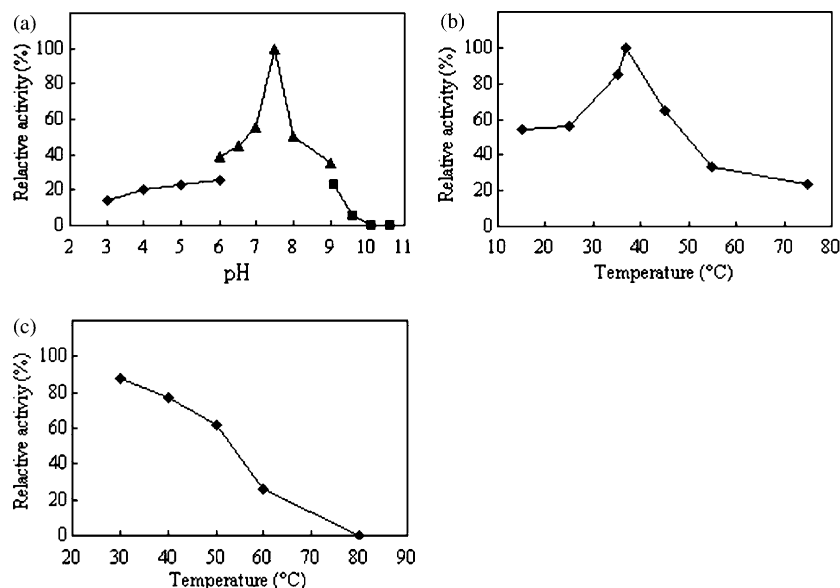

Figure 3. Effects of pH and temperature on the activity of AlgL. Experiments were carried out as described in the section Materials and methods. (a) Effects of pH: reactions were performed at 37 °C for 20 min in the following buffers: ♦, 50 mM citrate buffer (pH 3–6); ▲, 100 mM phosphate buffer (pH 6–9); ■, 100 mM glycine–NaOH (pH 9.1–10.6). (b) Optimal temperature: reactions were performed for 20 min at various temperatures in 100 mM phosphate buffer (pH 7.5). The activity at 37 °C was relatively taken as 100%. (c) Thermal stability: after preincubation of the enzyme at various temperatures for 60 min, the remaining activity was measured. The activity of the enzyme without preincubation was relatively taken as 100%.

Table 1. Effects of chemical reagents on the lyase AlgL.

Reagent	Concentration[a] (mM)	Relative activity (%)
None		100
NaCl	100	339
	300	341
KCl	1	415
$MgCl_2$	1	314
$BaCl_2$	1	418
$ZnCl_2$	1	79
$CaCl_2$	1	247
$NiCl_2$	1	73
$AlCl_3$	1	232
$CuSO_4$	1	82
$FeCl_3$	1	0
$MnCl_2$	1	316
EDTA	1	109
SDS	1	0

[a]The final concentration of each reagent in the assay mixture.

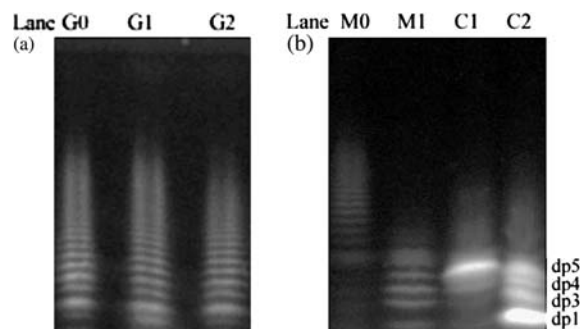

Figure 4. FACE analysis of the substrate-specificity of AlgL. Lane G0–2, samples corresponding to reacting system with G blocks as substrate, incubated at 37 °C for 0, 30 min and 12 h, respectively. Lane M0-1, samples correspond to reacting systems with M blocks as the substrate, incubated at 37 °C for 0, 30 min, respectively. Lane C1, sample corresponding to polymannuronate oligomers with dp of 5 as standard sample. Lane C2, sample corresponding to polymannuronate oligomers with dp of 1, 3, 4, 5 as standard sample.

difficulties in the detection of the carbohydrates in the gels (Koketsu *et al.* 2000). Furthermore, after polyacrylamide gel was stained by Alcian blue, only those oligosaccharides with dp more than 8 could be viewed directly on the gels when about 10 μg samples were loaded (data not shown). In contrast, the FACE method in our assay showed much more sensitivity. As those oligosaccharides with dp less than 15 that contained the original reducing end can be labelled with sulphonated fluorophores and viewed directly on the gels when about 3.3 μg samples were loaded (Figure 4). In addition, FACE method could be used to analyse neutral oligosaccharides that contained the original reducing end. ANDS had two sulphonic acid groups, which gave the derivatized carbohydrates a greater net negative charge to improve their migration and resolution.

Conclusions

A novel alginate lyase gene *algL* of *Pseudomonas* sp. QD03 was cloned corresponding to the conserved regions of M-specific lyases by degenerate PCR and long range-inverse PCR (LR-IPCR). The recombinant AlgL could degrade highly acetylated alginate of *P. aeruginosa* FRD1 (approximately 0.54 mol of *O*-acetyl

Figure 5. PAGE analysis of hydrolysis products from bacterial alginate of *P. aeruginosa* FRD1 by AlgL. Lane 1, bacterial alginate of *P. aeruginosa* FRD1 without enzyme treatment; Lane 2, sample corresponding to bacterial alginate of *P. aeruginosa* FRD1 as the substrate incubated with 25 U of AlgL at 37 °C for 30 min.

group per mol of alginate). Furthermore, Ca^{2+} observably enhanced the activity of AlgL, and Zn^{2+} hardly inhibited the lyase activity in the level of human sputa. The results indicated that it might be possible to use alginate lyases AlgL as an adjuvant therapeutic agent for the treatment of diseases associated with *P. aeruginosa* infection. The successful identification of the new *algL* gene from soil microbe community constitutes a new approach for accessing and exploring novel lyases with high activity against highly acetylated bacterial alginate from environmental microbial community.

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