

Article



Genome Analysis of a Potential Novel *Vibrio* Species Secreting pH- and Thermo-Stable Alginate Lyase and Its Application in Producing Alginate Oligosaccharides

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Abstract: Alginate lyase is an attractive biocatalyst that can specifically degrade alginate to produce oligosaccharides, showing great potential for industrial and medicinal applications. Herein, an alginate-degrading strain HB236076 was isolated from Sargassum sp. in Qionghai, Hainan, China. The low 16S rRNA gene sequence identity (<98.4%), ANI value (<71.9%), and dDDH value (<23.9%) clearly indicated that the isolate represented a potential novel species of the genus Vibrio. The genome contained two chromosomes with lengths of 3,007,948 bp and 874,895 bp, respectively, totaling 3,882,843 bp with a G+C content of 46.5%. Among 3482 genes, 3332 protein-coding genes, 116 tRNA, and 34 rRNA sequences were predicted. Analysis of the amino acid sequences showed that the strain encoded 73 carbohydrate-active enzymes (CAZymes), predicting seven PL7 (Alg1-7) and two PL17 family (Alg8, 9) alginate lyases. The extracellular alginate lyase from strain HB236076 showed the maximum activity at 50 °C and pH 7.0, with over 90% activity measured in the range of 30–60 °C and pH 6.0–10.0, exhibiting a wide range of temperature and pH activities. The enzyme also remained at more than 90% of the original activity at a wide pH range (3.0-9.0) and temperature below 50 °C for more than 2 h, demonstrating significant thermal and pH stabilities. Fe²⁺ had a good promoting effect on the alginate lyase activity at 10 mM, increasing by 3.5 times. Thin layer chromatography (TLC) and electrospray ionization mass spectrometry (ESI-MS) analyses suggested that alginate lyase in fermentation broth could catalyze sodium alginate to produce disaccharides and trisaccharides, which showed antimicrobial activity against Shigella dysenteriae, Aeromonas hydrophila, Staphylococcus aureus, Streptococcus agalactiae, and Escherichia coli. This research provided extended insights into the production mechanism of alginate lyase from Vibrio sp. HB236076, which was beneficial for further application in the preparation of pH-stable and thermo-stable alginate lyase and alginate oligosaccharides.

Keywords: alginate lyase; Vibrio sp. HB236076; genome; oligosaccharide; antimicrobial activity

1. Introduction

Alginate is a class of polysaccharides widely distributed in brown seaweeds such as *Laminaria* and *Sargassum*, with a content of approximately 30–60% of their dry weight [1]. As a water-soluble acidic polysaccharide, it is constituted by two isomer residues, α -L-guluronic acid (G) and β -D-mannuronic acid (M). These two residues are connected by 1, 4-glycosidic bonds to contribute three different blocks, namely polyM, polyG, and polyMG blocks [2]. Alginate, with high viscosity and gelling properties, has enormous potential for application in the food, cosmetics, and pharmaceutical industries [3]. Meanwhile, the low water solubility and high solution viscosity limit its application when high concentrations are needed, especially in food products.



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Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). Alginate oligosaccharides (AOS) are molecules formed by the decomposition of alginate with a degree of polymerization (DP) of 2 to 25. They have shorter chain lengths and thus improve water solubility compared to higher molecular-weight alginates with the same monomers. Many pathways can be used to degrade alginate into AOS with low DP, including physical, chemical, and enzymatic degradation. Compared with physical and chemical methods, the enzymatic methods have the advantages of high efficiency, specificity, safety, and decreased byproducts [4]. Alginate lyases play a key role in alginate degradation through an enzymatic route and can be used for the directional preparation of AOS with a specific monosaccharide and DP [5]. Owing to a double bond between C4 and C5 at the non-reducing end, AOS exhibit higher biological activities [6]. Recently, AOS have gained growing attention because of their important biological functions such as antimicrobial, antioxidant, prebiotic, antitumor, anticoagulant, immunomodulatory, and plant root growth-promoting activities [6–11]. Due to these characteristics, AOS have broad application potential in the agricultural, food, and pharmaceutical industries [12].

Alginate lyases can depolymerize alginate into oligosaccharides or monosaccharides by cleaving glycosidic bonds through a β -elimination reaction [13]. According to substrate specificity, alginate lyases can be divided into polyG-specific lyases (EC 4.2.2.11), polyMspecific lyases (EC 4.2.2.3), and polyMG-specific lyases (EC 4.2.2), which can degrade polyG, polyM, and polyMG blocks of alginate, respectively [14]. In the Carbohydrate-active enzymes (CAZymes) database (http://www.cazy.org/ (accessed on 15 June 2024)), alginate lyases are categorized into 15 polysaccharide lyase (PL) families (PL5, 6, 7, 14, 15, 17, 18, 20, 31, 32, 34, 36, 38, 39, and 41) based on their amino acid sequences, which imply significant structural diversity of the catalytic sites [15]. Alginate lyases come from a wide range of sources, including marine mollusks, bacteria, fungi, and marine brown alga [16]. Numerous studies have reported the ability of bacteria to degrade alginates, such as *Alteromonas* [17], Pseudoalteromonas [18], Paenibacillus [19], Microbulbifer [20], and Zobellia [21]. As is well known, members of the genus Vibrio can also degrade alginate due to their alginolytic systems, which include diverse alginate lyases. A variety of alginate lyases have been explored from Vibrio species, for instance, V. harveyi AL-128 [22], V. splendidus 12B01 [23], V. xiamenensis QY104 [24], Vibrio sp. dhg [25], and Vibrio sp. W13 [26].

In this study, an alginate-degrading strain HB236076 was isolated from *Sargassum* sp. in Qionghai, Hainan, China. Based on the 16S rRNA gene and genome sequence analyses, the strain was assumed to represent a potential novel species of the genus *Vibrio*. The general characteristics of its genome sequence were reported, and genome annotation showed that it contained abundant alginate lyases. In addition, we further investigated the properties of alginate lyase in culture supernatant and the AOS produced by enzyme degradation.

2. Results

2.1. Screening of Strain HB236076

The screening results obtained by the agar plate method showed that strain HB236076 from *Sargassum* sp. exhibited significant alginate lyase activity. Under the action of 1 M CaCl₂, a white ring produced by gelation reaction on the plate showed that alginate lyase was secreted (Figure S1).

2.2. Genome Specifics

The complete genome of strain HB236076 was determined, and two circular chromosomes were obtained, with the NCBI GenBank accession numbers CP162601 and CP162602. A total of 65,029 clean reads with an average read length of 8717 bp, totaling 566.9 Mb, were analyzed, giving 146× coverage depth. Strain HB236076 presented two chromosome genomes of 3,007,948 bp and 874,895 bp, totaling 3,882,843 bp, with a G+C content of 46.5%. A total of 3482 genes were predicted, including 3332 protein-coding genes, 116 tRNA, and 34 rRNA (12, 11, 11 for 5S, 16S, and 23S rRNAs, respectively) sequences. The general features of strain HB236076 genome are shown in Figure 1 and Table S1.



Figure 1. Circos maps of the two chromosome genomes of strain HB236076. (a) Chromosome 1; (b) chromosome 2. The maps were divided into 7 circles from outside to inside, namely, markers of genome size (5 kb per scale), genes on the positive strand, genes on the negative strand, repetitive sequences, genes of tRNA (blue) and rRNA (purple), GC content (buff: above mean; blue: below mean), and GC-skew (dark gray: G higher than the C; red: G lower than the C).

The gene functions were classified with KEEG databases, and a total of 2123 proteins were annotated. ABC transporters with a total of 170 genes accounted for 8.0% of the annotated genes and comprised the highest proportion in the KEGG pathway, followed by biosynthesis of amino acids (5.2%), two-component system (4.9%), carbon metabolism (3.9%), purine metabolism (3.7%), and pyrimidine metabolism (2.8%). Bioinformatics analysis using antiSMASH predicted four antibiotic resistance proteins, namely fluoroquinolone, penam, macrolide, and tetracycline antibiotics.

2.3. Identification of Strain HB236076

After culturing on MA at 30 °C for 2 d, colonies of strain HB236076 were beige, circular, regular, smooth, and 1 mm in diameter. A nearly complete 16S rRNA gene sequence (1474 bp) by PCR amplification was obtained, and the complete sequence (1552 bp) was determined from the genome with GenBank No. PP999765. Phylogenetic analysis indicated that the isolate belonged to the genus Vibrio. The closest phylogenetically related species were V. maritimus R-40493^T, V. variabilis R-40492^T, V. japonicus JCM 31412^T, and V. sinaloensis CAIM 797^T, with 98.4, 98.1, 98.0, and 98.0% similarities, respectively. The other 16S rRNA gene sequence similarities were under 98.0%. The phylogenetic tree reconstructed using the neighbor-joining method showed that the strain clustered together with V. halioticoli NBRC 102217^{T} (with 16S rRNA gene similarity 97.4%), significantly supported by a bootstrap value of 63%, which indicated that strain HB236076 had the closest phylogenetic affinity to V. halioticoli (Figure 2). The low levels of sequence similarities are much lower than the 98.65% threshold for recognizing a novel species, indicating that this strain may represent a novel species of the genus Vibrio [27]. At the genomic level, strain HB236076 exhibited ANI values of 70.8–71.9% and dDDH values (the recommended results from formula 2) of 22.3–23.9% with the upper five closest relatives (Table S2), which were lower than the threshold values of the species boundary (ANI 94–96% and dDDH 70%) [28,29]. Hence, clearly, the results of the phylogenetic and genotypic analysis clearly indicated that strain HB236076 represented a potential novel species of the genus Vibrio. It was temporarily named Vibrio sp. HB236076 in this article.



Figure 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences between strain HB236076 and related species of the genus *Vibrio* by comparison of 1478 nucleotides. Bootstrap values (1000 replicates) are shown as percentages at each node for values; only values > 50% are shown. *Grimontia hollisae* ATCC 33564^T was used as an outgroup. The scale bar represents 0.01 nucleotide substitutions per position.

Members of the genus *Vibrio*, widely distributed in marine environments, are an important group for alginate degradation, from which a large number of alginate lyases have been elucidated. Algb from marine bacterium *Vibrio* sp. W13 was identified as a novel endolytic alginate lyase that produced oligosaccharides with DP2–5 in an endolytic manner [26]. AlyH1 from *V. furnissii* H1 was a novel and potential candidate in the application of alginate lyase with two functional catalytic domains that were synergistic in alginate degradation [31]. Herein, *Vibrio* sp. HB236076, derived from *Sargassum* sp. in the South China Sea, provided a new source for alginate lyase.

2.4. Genetic Basis of Alginate Degradation

In order to elucidate the related polysaccharide-degrading enzymes, the CAZymes were predicted using the dbCAN and InterPro databases. Strain HB236076 had 73 CAZymes, including 25 glycoside hydrolases (GHs), 28 glycosyl transferases (GTs), 11 polysaccharide lyases (PLs), 4 carbohydrate esterases (CEs), 3 carbohydrate-binding modules (CBMs), and 2 auxiliary activities (AAs). As shown in Table 1, nine of the putative PLs were predicted to encode alginate lyases, named Alg1–9, which indicated that Vibrio sp. HB236076 had powerful alginate-degrading ability. The open reading frames (ORFs) consisted of 840, 1959, 1032, 858, 747, 1029, 909, 2163, and 2163 bp nucleotides and encoded 279, 652, 343, 285, 248, 342, 302, 720, and 720 amino acids, respectively. The enzymes of Alg1-7 belonged to the PL7 family, and the other two enzymes belonged to the PL17 family. The sequence alignment results showed that Alg7 had the lowest identity among all the nine alginate lyases, with the highest sequence identity (47.9%) with polysaccharide lyase PL7 (WP_318074641.1) from unclassified Vibrio. Generally speaking, bacteria can secrete a series of lyases or glycosidases to depolymerize polysaccharides and produce oligosaccharides or monosaccharides that can be utilized by cells. CAZymes typically account for no more than about 2% in most bacterial genomes and seldom exceed 5% in the genomes of bacteria that specialize in carbohydrate degradation [32]. Overall, the number of CAZymes produced by the strain is not significant, accounting for 2.2%, but it has abundant alginate lyases, accounting for 12.3% of the CAZymes, indicating that the isolate has a strong ability to degrade alginate.

Enzymes	Accession No.	PL Family	Length (aa)	MW (Dal)	Signal Protein	Sequence Comparison ¹			
						PI	Sequence Identity (%)	Reference Alginate Lyase	
Alg1	XDK25489.1	PL 7	279	30,235.57	Yes	3.90	61.9	GAA4099934.1	
Alg2	XDK26069.1	PL 7	652	71,934.82	Yes	5.66	79.6	WP_231565442.1	
Alg3	XDK26066.1	PL 7	343	38,020.27	Yes	6.17	76.0	WP_102470889.1	
Alg4	XDK25655.1	PL 7	285	32,002.96	Yes	6.80	97.2	ABB36771.1	
Alg5	XDK24838.1	PL 7	248	28,306.44	Yes	4.50	97.0	ABB36772.1	
Alg6	XDK24840.1	PL 7	342	37,836.15	Yes	6.60	60.6	WP_334550621.1	
Alg7	XDK24841.1	PL 7	302	33,260.67	Yes	4.51	47.9	WP_318074641.1	
Alg8	XDK24833.1	PL 17	720	80,516.22	No	5.39	65.1	CAH6913425.1	
Alg9	XDK24834.1	PL 17	720	80,465.18	No	5.68	63.2	WP_252024937.1	

Table 1. Characteristics of the alginate lyases identified in the genome of strain HB236076.

¹: The reference sequences did not include the sequence from strain HB161653 we submitted.

As shown in Figure 3a, Alg1, 3–7 had only one domain but had different modularity; Alg2 had three domains, namely a catalytic domain of alginate lyase and two F5_F8 type_C domains generally known as the discoidin domain [33]; Alg8 and Alg9 had two structural domains, namely alginate lyase and Hepar_II_IIII domains, which meant the two enzymes of PL17 family could not only degrade alginate but also had the ability to degrade heparin and heparin sulfate. The signal peptide was predicted in Alg1–7, indicating that the seven alginases might be extracellular enzymes, while Alg8 and Alg9 did not have signal peptides and might be intracellular enzymes. In the phylogenetic tree of alginate lyases, including PL7 and PL17 families (Figure 3b), Alg1–7 were clearly located in the clade of the PL7 family, while Alg8 and Alg9 formed a distinct branch of the PL17 family and clustered in the same clade. Alg1 was classified within subfamily 4 of the PL7 family; Alg2, 3, and 6 were classified within subfamily 5 of the PL7 family; Alg4, 5, and 7 were classified within subfamily 6 of the PL7 family. The clustering results were consistent with the results predicted by the CAZy database.

According to the analysis of amino acid sequence, the multiple sequence alignments of Alg1–7 and nine well-characterized alginate lyases of the PL7 family are shown in Figure 4. Alg1–7 contained three highly conserved regions of the PL7 family, namely, R(S/T/V)ELR, Q(I/V)H, and YFKAG, which had an indispensable influence on the substrate binding and catalytic activity of the enzymes [18,33]. It is reported that the conserved domain of the region "QIH" means that it is more inclined to degrade polyG, while "QVH" means that it is more inclined to use polyM as the substrate [34]. The alginate lyases containing the QIH region, such as Alg2951 from *Alteromonas portus* [35] and ALY-1 from *Corynebacterium* sp. strain ALY-1 [36], degraded polyG in activity assays. Alginate lyases containing the QVH region, such as alginate lyase AlyVOA and AlyVOB from *Vibrio* sp. O2 [37] and A9m from *Vibrio* sp. JAM-A9m [38], preferred polyM as the substrate. According to the Q(I/V)H region, Alg1–3, 6 containing QIH were predicted to be polyG-preferred alginate lyases, while Alg4, 5, 7 containing QVH might be polyM-preferred alginate lyases. It is speculated that strain HB236076 could degrade polyG, polyM, and alginate.

In addition to the enzymes responsible for degrading alginate, other polysaccharidedegrading enzymes were predicted, including two α -amylases of the GH13 family, one β -galactosidase of the GH2 family, one glucosylceramidase of the GH30 family, one peptidoglycan hydrolase of the GH73 family, and one 1,4- α -glucan-branching enzyme (GlgB) of the GH13 family (Table S3). α -Amylases are a well-known type of endo-amylases and have been found in a large number of microorganisms, such as *Bacillus amyloliquefaciens*, *Bacillus licheniformis, Anoxybacillus beppuensis*, and *Pyrococcus woesei* [39], which have proved to be of great value in all starch-based industries. β -Galactosidase is a glycoside hydrolase that primarily breaks down lactose into glucose and galactose in bacteria, entering the glycolysis process to provide energy and carbon sources. GlgB is responsible for the transfer of chains of α -1,4-linked glucosyl residues to other similar chains (in new α -1,6 **(a)** Alginate lyases of strain HB236076 0 100 aa 200 aa 300 aa 400 aa 500 aa 600 aa 700 aa 800 aa signal peptide Alg 1 alginate_lyase2 signal peptide Alg 2 alginate_lyase2 F5_F8_type_C F5_F8_type_C signal peptide Alg 3 alginate_lyase2 signal peptide alginate_lyase2 Alg 4 signal peptide alginate_lyase2 Alg 5 signal peptide alginate_lyase2 Alg 6 signal peptide alginate_lyase2 Alg 7 alginate_lyase Hepar_II_III Alg 8 Alg 9 Hepar_II_III alginate_lyase **(b)** XDK24840.1 Alg6 99 AIY68670.1 alkaline alginate lyase Agarivorans sp. L11 XDK26066.1 Alg3 96 73 PL7_5 CAZ98266.1 alginate lyase family PL7 Zobellia galactanivorans 55 XDK26069.1 Alg2 37 100 AAA25049.1 alginate lyase Klebsiella pneumoniae XDK25489.1 Alg1 PL7_4 32 78 OTA00986.1 alginate lyase Trichoderma parareesei 44 CU70527.1 alginate lyase 2 Catenulispora acidiphila DSM 44928 35 59 BAD16656.1 alginate lyase Sphingomonas sp. A1 PL7_1 34 27 AAG04556.1 hypothetical protein PA1167 Pseudomonas aeruginosa PAO1 11 ACO79344.1 alginate lyase Azotobacter vinelandii DJ 90 ABS59291.1 alginate lyase precursor Streptomyces sp. ALG5 WP 053404615.1 alginate lyase Persicobacter sp. CCB-QB2 33 97 PL7_3 84 CAZ95239.1 alginate lyase family PL7 Zobellia galactanivorans 99 AXO88466.1 polysaccharide lyase family 7 protein Pseudomonas parafulva 22 100 AAY93971.1 conserved hypothetical protein *Pseudomonas protegens* Pf-5 XDK24838.1 Alg5 PL7 2 100 100ABB36772.1 alginate lyase AlyVOB Vibrio sp. O2 XDK24841.1 Alg7 USW90871.1 alginate lyase V Vibrio pelagius PL7 6 98 QOP59290.1 alginate lyase Psychromonas sp. 89 XDK25655.1 Alg4 100 100 ABB36771.1 alginate lyase AlyVOA Vibrio sp. O2 66 ABD82539.1 putative alginate lyase Saccharophagus degradans 2-40 <u>99</u> CAZ96774.1 Alginate lyase family PL17 Zobellia galactanivorans 100 UVF39826.1 AlyRM4 exo-alginate lyase Rhodothermus marinus **PL17** XDK24833.1Alg8 98 XDK24834.1 Alg9 98 100 UQN45019.1 heparinase II/III family protein Agarivorans sp. B2Z047 ABG42142.1 Poly(beta-D-mannuronate) lyase PL6 Paraglaciecola sp. T6c PL6

0.1

Figure 3. (a) Domain architectures of the nine alginate lyases of strain HB236076. (b) Neighborjoining phylogenetic tree of alginate lyases based on the predicted amino acid sequences. Bootstrap values (1000 replicates) are shown as percentages at each node for values. The scale bar represents 0.1 nucleotide substitutions per position. Putative alginate lyases of strain HB236076 are highlighted in bold. Poly (beta-D-mannuronate) lyase (ABG42142.1) from the PL6 family was used as an outgroup.

Alg1/1-264	1 TOGOAFAAPSDDFDLSOWKLTLPVSEYAYFGSGDDDDAAEILPSDCTGNNYSGSGIDEGFEDTNYFYSDSSGAMVFVTPLD	81
Alg2/1-278	1 ATPKAGNTPGQNFVLTTWYLSQPFDHDNDGKPDNVYEWDLANGYQHPEIFYTADDGGVLVFKSYIK	65
Alg3/1-318	1 TRAPGQQF	59
Alg4/1-260	1 NPSGQL GE PANYSQFAN I LNAS ALVEKMA	67
A105/1-231		62
Ala6/1-200		60
Alg01-000		00
Alg//1-249		09
BAA83339.1/1-22/	1AAAAE	63
BAP05660.1/1-277	1 TVLATATISNAQDKKSKSKTAKIDWSHWTVIVPEENPDKPGKPYSLGYPEILNYAEDKIASKYMYDDPKDKSVVFYAFPS	80
AAA25049.1/1-282	1 APGDKFYRNSDFFTLSDAGG	58
WP053404615.1/1-2	2 1 • APGTAQI PSDLMSNCN • • • • • • • • • • • • • • • • • •	56
AAG04556.1/1-223	1QGRPAITISTSQLQRDYRSDY FQRTADGIRFWVPVNG	49
RAD16656 1/1-242	1 AASSSSSSSSSS DPAAAPGKNEDI SHWKLOL PDANTTELSSANLGLG	69
115000871 1/1-267	1 NAS, DKAADADYEOLODVI KVS	68
CA709222 4/4 242		60
CA290200.1/1-313	TAMKATIAT SELTP	29
CA295239.1/1-253	1 TLPISGNSMASVLGITANTWKINSFIGSPGSSATYYDDITDASGISYNTYSDDNYHYTDGEWVYFRCYRG	70
Alg1/1-264	82 G · · GASTL NSSYYRSELRELYDWSACDSTGTAN · · · · · · · · · · · · · · · · · · ·	141
Alg2/1-278	66 G · · VRTSKNTKYARTEMREMLRAGDKSISTKGVNKNNWVFSSAPIEDQKAAGGVDGVLEATLKIDHTTTTG · · · · · · ELGE · · · VGRFIIG · · · · · · ·	145
Ala3/1-318	60 A I TTKNSKN ARSEL ROMARGADES I GTH. DAKNNWALSSHPOAD TESA I GGTLEATLKYNHYSLHAKYPEKYP AFSVYYG	138
Ala4/1-260	68 NDHC ENEL BYOKNER. TDI PDHEY	90
Ala5/4-224		05
Algor-231		35
Algo/1-309	70 APTTPNSKNARSELRAMYAEAYD.EPANNFVLASHPDAEKYGSTGGQLSATVSVDWVSQSGDANKTGAYSVVTG	142
Alg7/1-249	70	88
BAA83339.1/1-227	64NGVTTSGSGYQRSELREMTDGGEE	119
BAP05660.1/1-277	81 G · · · VTTANTHY \$ RSEL RE TME T GSNKVNWT · · · · · · · · · · · · · · · · · · ·	109
AAA25049.1/1-282	59 G. AKTSKNTTY I RSELREMLRKGDTSI ATOGVSRNNWVLSSAPL SEQKKAGGVDGTLEATLSVDHVTTTGVNWQVGRVI I G	138
WP053404615.1/1-2	2 57 SNNG TTPNSSY RSELRERKEDGSA	112
44604556 1/1-223	50 SHTRNSFERRET BETI SSCRDYN. WRYARADNM FATI RIFAVDSTRRMIIG.	101
DAD42262 4/1 0/0		07
BAD10030.1/1-242	10 GG IT ANSSTERSEL DESNSKY WYG	97
USW908/1.1/1-26/	69 K	89
CAZ98266.1/1-313	60 GITSRTSSNTRTELGO	113
CAZ95239.1/1-253	71 L GGSANSQNÍ RVELREMDNGNLASWT GD	98
Alg1/1-264	142	174
Alo2/1-278		166
4103/1-318		173
Ala 111-260	AT THANNELMUDAASMANS TSKADELTS AVALUES TO TO TOTUNYOUDI LOVAMEDNACYVCUGMATTANNAVICKASS	160
Alg4/1-200		100
Algo/1-231	96 LIGEVDETIF DVHDVISN	149
Alg6/1-309	143 NEPLKIAYRKLPGHD	163
Alg7/1-249	89 VLTASFKPIGIEAAMSSTSSAN.DAVTFAQVHNKGEDDSGTGYIPHPLVRIVYEANRNNLYGHYWAIIKNNSLDCS	163
BAA83339.1/1-227	120 VT SDYKL NT VFE GKFVVS GGK I KVY YN 1	177
BAP05660.1/1-277	110 AKGGKMRGTYAIDDISKEPDGKYSRVII AQIHGVLTDEQ	174
AAA25049.1/1-282	139 DEPIRLYYRKLPHIO	159
WP053404615.1/1-2	113	178
AAG04556 1/1-223		111
PAD46656 4/4 242		424
BAD10000.1/1-242		131
0311900/1.1/1-20/	30 NL GAETAPTAPTASVANTDAARNOATTTE OVINAGVSADFPDOVSGOTTPIPE VKVVTEAEKSGRNOWIWAVTKNAAVNC	1/1
CA298200.1/1-313	114 GHE SHE SHE	137
CAZ95239.1/1-253	99 SGTHTMEWTVQVNQLPQDTDGDGGVLCF Q1H GPSKNSDGVEVDDVVRVQFIGEENQS	156
Alg1/1-264	175 GNN FDL E FGL VPG	187
Alg2/1-278	167 TGTVYFAHENTLK	226
Alg3/1-318	174 TGSVFWNYERNLAKNDPNRMDIAYPVWGNTWENKHK 2	242
Alg4/1-260	169 GKK. NKDKEMCRADVAYSKIDLGP APQGKSTD.FTITVG	205
Alo5/1-231	150	181
AI06/1-309	164 LOST YWGYEL NDRKAOOKSKI RKDLRSNVWG.K. YNI KAG	240
Ala7/4-240		106
ANJ11-243		130
DAA03339.1/1-227		191
BAPU3000.1/1-2//	1/2 KINVLNUPTREMLSENAWUUUEUK·································	
AAA25049.1/1-282		222
WP0534046151/1-2	160 KGSVYFAHE . PRKG	230
111 000101010.0112	160 KGSVYFAHE - PRKG · · · · · · · · FGDEQWYEMI GTLQPSHGNQTAAPTEP · EAGTAL GETFSYRI · · · · · · DATGNKLTVTLMRE · · · · · · GRP 2 9 179 · · · · · · · · · · · · · · · · · · ·	222 230 211
AAG04556.1/1-223	160 KGSVYFAHE - PRKG · · · · · · · · · · · FGDEQWYEMI GTLQPSHGNQTAAPTEP - EAGTAL GETFSYRI · · · · · · DATONKLTVTLMRE · · · · · · GRP 179 · · · · · · · · · · · · · · · · · · ·	222 230 211 191
AAG04556.1/1-223 BAD16656.1/1-242	160 KGSVYFAHE - PRKG	222 230 211 191 208
AAG04556.1/1-223 BAD16656.1/1-242 USW90871.1/1-267	160 KGSVYFAHE - PRKG DATONKLTVTLMRE GRP 179 GKLAE AYANGSAAAYLI KDGGNDYVMDLNYDQS 112 QAAPLVKLLYQLRLDQGRVQALVRE RPDDGGTRAYTLMDG IPLGVP SYRIGVSRSGLLSVSVNGSALEQQLDPOWAYQG 112 132 TNAPPLVKAVFQDQQLDWQNSDGTGSDVHNYFTGIKLGUYNME I RVTDG VAYVTMNGDTRSVDFVGKDAGWKN 112 172 GSKSGNKGTEECKN AYLLPIAN	222 230 211 191 208 209
AAG04556.1/1-223 BAD16656.1/1-242 USW90871.1/1-267 CAZ98266.1/1-313	160 KGSVYFAHE - PRKG	222 230 211 191 208 209 213
AAG04556.1/1-223 BAD16656.1/1-242 USW90871.1/1-267 CAZ98266.1/1-313 C4Z95239.1/1-253	160 KGSVYFAHE - PRKG	222 230 211 191 208 209 213 220
AAG04556.1/1-223 BAD16656.1/1-242 USW90871.1/1-267 CAZ98266.1/1-313 CAZ95239.1/1-253	160 KGSVYFAHE - PRKG FGDEQWYEMI GTLQPSHGNQT AAPTEP - EAGTAL GETFSYRI DATGNKLTVTLMRE GRP 179 GKLAEAYANGSAAAYLI KDGGNDYMDLNYDQS 112 KDGGNDYMDLNYDQS 1132 132 TNAPPLVKAVFQDQGLDMQVKQNSDGTGSDVHNYFTGLK GDLYNME I RVTDG - VAYVTMNGDTRSVDFVGKDAGWKN 112 AKEG - TOKFDI YVGG 172 GSKSGNKGTEECKN AYLKLPIAPI AKEG - TOKFDI YVGG 1132 173 GSKSGNKGTEECKN AYLKLPIAPI AKEG - TOKFDI YVGG 1132 174 GSKSGNKGTEECKN AYLKLPIAPI AKEG - TOKFDI YVGG 1132 175 GSKSGNKGTEECKN AYLKLPIAPI AKEG - TOKFDI YVGG 1132 174 GSKSGNKGTEECKN AYLKLPIAPI AKEG - TOKFDI YVGG 1132 175 GSKSGNKGTEECKN AYLKLPIAPI AKEG - TOKFDI YVGG 1132 175 GSKSQLKLKISGYVELFMNGSSK RWDYSTAWG YDMSVVGPTA TSYPEEP - EDGIAL GEEFSYEI NVYEGIMYLTFSSE 157 SGSVKLKISGYVTEEQGGS 0TFSGYSLDTTYNCKLVYSGGYVELFMNGSSVFRKKMEVDDLSEN 0TFSGYSLDTTYNCKLVYSGGYVELFMNGSSVFRKKMEVDDLSEN	222 230 211 191 208 209 213 220
AAG04556.1/1-223 BAD16656.1/1-223 USW90871.1/1-267 CAZ98266.1/1-313 CAZ95239.1/1-253 Alp1/1-264	160 KGSVYFAHE - PRKG FGDEQWYEMI GTLQPSHGNQTAAPTEP - EAGTAL GETFSYRI DATONKLTVTLMRE GRP 179 GKLAEAYANGSAAAYLI KDGGNDYVMDLNYDQS 112 112 QAAPLVKLLYQLRLDQGRVQALVRE RPDDGGTRAYTLMDG IPLGOP SYRI GVSRSGLLSVSVNGSALEQQLDPQWAYQG 113 132 TNAPPLVKAVFQDGQLDMQVKQNSDGTGSDVHNYFTGIK GDLYNME IRVTDG VAYVTMNGDTRSVDFVGKDAGWKN 117 138 KGSVFWNYE INTKGDNSK RWDYSTAWG YDMSVVGPTA TSYPEEP - EDGTALGEFSYEI NVYEGIMYLTFSSE 138 TDEWSYT I EVERKKI SI SVTYGGETVTKSVT I GEGDMDDDWD DDT VEKAGUWARABUKSSGGET QTFSGYSL VKETAUDVSUED	222 230 211 191 208 209 213 220
AAG04556.1/1-223 BAD16656.1/1-242 USW90871.1/1-267 CAZ98266.1/1-313 CAZ95239.1/1-253 Alg1/1-264 Alc24.278	160 KGSVYFAHE - PRKG FGDEQWYEMI GTLOPSHGNQTAAPTEP - EAGTAL GETESYRI DATONKLTVTLMRE GRP 179 GKLAEAYANGSAAAYLI KDGGNDYMDLNYDOS GKLAEAYANGSAAAYLI KDGGNDYMDLNYDOS 112 QAAPLVKLLYQLRLDQGRVQALVRERPDDGGTRAYTLMDGIP GOPFSYRI GVSRSGLLSVSVNGSALEQQLDPQWAYQG GKLAEAYANGSAAAYLI KDGGNDYWNLNYDOS 12 TNAPPLVKLYQLRLDQGRVQALVRERPDDGGTRAYTLMDGIP GOPFSYRI GVSRSGLLSVSVNGSALEQQLDPQWAYQG GKKGNKNN GKKGNKKN 12 TNAPPLVKAVFQDGQLDMQVKQNSDGTGSDVHNYFTGIKGDYMNE IRVTDG-VAYYTMNGDTRSVDFVGKDAGWKN AKEG.TDKFDIYVGG GKKGNKKN 172 GSKSGNKGTEECKN AYLKLPIAPI AKEG.TDKFDIYVGG GKKGVKNN 173 KGSVFWNYE INTKGDNSK RWDYSTAWG YDMSVVGPTA TSYPEEP. EDGIALGEFSYEI NVYEGIMYLTESSE 157 SGSVKLKISGYTEEQGGS QIFSGYSLDTTYNCKLVYSGGYVELFMNGSSVFRKKMEVDDLSEN VKFTDLDVSHSD 188 TDEWSYT I EVEDKKISISVTYGGETVTKSVT I GEGDMDDDWLDDT YFKAGNAKSSGGSFT VKFTDLDVSHSD 2020 CVVVVVNSSOF CVVVVVNSSOF VKFTDLDVSHSD VVVVVNSSOF	222 230 211 191 208 209 213 220 264
A4G04556.1/1-223 BAD166556.1/1-242 USW90871.1/1-267 CAZ98266.1/1-313 CAZ95239.1/1-253 Alg1/1-264 Alg2/1-278	160 KGSVYFAHE - PRKG FGDEQWYEMI GTLQPSHGNQTAAPTEP - EAGTAL GETESYRI DATONKLTVTLMRE GRP 179 GKLAEAYANGSAAAYLI KDGGNDYMDLNYDQS 112 QAAPLVKLLYQLRLDQGRVQALVRERPDDGGTRAYTLMDG IPLGQPFSYRI GVSRSGLLSVSVNGSALEQQLDPQWAYQG 113 12 TNAPPLVKAVFQDGQLDMQVKQNSDGTGSDVHNYF GT KLGQLYNME IRVTDG - VAYVTMNGDTRSVDFVGKDAGWKN 112 132 TNAPPLVKAVFQDGQLMQVKQNSDGTGSDVHNYF GT KLGQLYNME IRVTDG - VAYVTMNGDTRSVDFVGKDAGWKN 112 133 GSKSGNKGTEECKN AYLKLPIAPI AKEG - TDKFDIYVGG 134 GSVFWNYE I NTKGDNSK RWDYSTAVWG YDMSVVGPTA TSYPEEP - EDGTAL GEEFSYEI NVYEGIMYLTFSSE 157 SGSVKLKI SGYVTEEQGGS OTFSGYSL DTTYNCKLVYSGGYVEL FMNGSSVFRKKME VDDLSEN 115 188 TDEWSYT I EVEDKKI SI SVTYGGET VTKSVT I GEGDMDDDWLDDT YFKAG W AQANKSSGSFT VKFTDLDVSHSD 227 DAVQVVDMSDS GYDVGGKYNYFKAGY NO NI SGDPDD YVQAT FYRI HKTHDQ	222 230 211 191 208 209 213 220 213 220 264 278
AAG04556.1/1-223 BAD16556.1/1-223 USW90871.1/1-267 CA298266.1/1-313 CA295239.1/1-253 Alg1/1-264 Alg2/1-278 Alg3/1-318	160 KGSVYFAHE - PRKG FGDEQWYEMI GTLQPSHGNQTAAPTEP - EAGTALGETFSYRI DATONKLTVTLMRE GRP 179 GKLAEAYANGSAAAYLI KDGGNDYVMDLNYDQS 112 QAAPLVKLLYQLRLDQGRVQALVRERPDDGGTRAYTLMDG IPLGOPFSYRIGVSRSGLLSVSVNGSALEQQLDPOWAYQG 112 132 TNAPPLVKAVFQDGQLDMQVKQNSDGTGSDVHNYFTGIKLGDLYNME IRVTDG VAYVTMNGDTRSVDFVGKDAGWKN 112 138 KGSVFWNYE INTKGDNSK RWDYSTAWG YDMSVVGPTA TSYPEEP - EDGTALGEFSYEI NVYEGIMYLTFSSE 138 KGSVFWNYE INTKGDNSK RWDYSTAWG YDMSVVGPTA TSYPEEP - EDGTALGEFSYEI NVYEGIMYLTFSSE 157 SGSVKLKISGYVTEEQGGS QTFSGYSLDTTYNCKLVYSGGYVELFMNGSSVFRKKMEVDDLSEN QTFSGYSLDTTYNCKLVYSGGYVELFMNGSSVFRKKMEVDDLSEN 188 TDEWSYT I EVEDKKISISVTYGGETVTKSVT I GEGDMDDDWLDDT YFKAGN AQANKSSGGSFT VKFTDLDVSHSD 227 DAVQVVDMSDS GYDVGGKYN YFKAGY NON I SGDPDD YVQATFYR I HKTHDQ 243 TVTYE I DLSKG-VODKDYPQ G YAKAANY YFKAGN YGCS-VSETHPWWGPGCQG-TGDFTVDKANGDYNSVTFSQLKLN-G	222 230 211 191 208 209 213 220 213 220 264 278 318
AAG04556.1/1-223 BAD16656.1/1-242 USW90871.1/1-267 CA298266.1/1-313 CA295239.1/1-253 Alg1/1-264 Alg2/1-278 Alg3/1-318 Alg4/1-280	160 KGSVYFAHE - PRKG FGDEQWYEMI GTLOPSHGNQTAAPTEP - EAGTAL GETESYRI DATONKLTVTLMRE GRP 179 GKLAEAYANGSAAAYLI KDGGNDYMDLNYDOS GKLAEAYANGSAAAYLI KDGGNDYMDLNYDOS 112 QAAPLVKLLYQLRLDQGRVQALVRERPDDGGTRAYTLMDGI PLGOPFSYRI GVSRSGLLSVSVNGSALEQQLDPQWAYQG GKLAEAYANGSAAAYLI KDGGNDYWNLNYDOS 12 QAAPLVKLLYQLRLDQGRVQALVRERPDDGGTRAYTLMDGI PLGOPFSYRI GVSRSGLLSVSVNGSALEQQLDPQWAYQG GKLAEAYANGSAAAYLI KDGGNDYWNDNNYTGGLSVSVNGSALEQQLDPQWAYQG 12 TNAPPLVKAVFDDGOLDMQVKQNSDGTGSDVHNYTFGLKLGUTNME IRVTDG-VAYYTMNGDTRSVDFVGKDAGWKN AKEG.TDKFDIYVGG. GKKSGNKGTEECKN. AYLKLPIAPI 172 GSKSGNKGTEECKN. AYLKLPIAPI AKEG.TDKFDIYVGG. GKKSGNKKTISTSVFVGFTA TSYPEEP.EDGIALGEFSYEI NVYEGIMYLTFSSE 173 KGSVFWNYE INTKGDNSK. RWDYSTAWG. YDMSVVGPTA TSYPEEP.EDGIALGEFSYEI NVYEGIMYLTFSSE GHK 7 175 SGSVKLKISGYTEEQGGS QTFSGYSLDTTYNCKLVYSGGYVELFMNGSSVFRKKMEVDDLSEN QTFSGYSLDTTYNCKLVYSGGYVELFMNGSSVFRKKMEVDDLSEN YKFTDUVSGGSFT VKFTDUVSHSD 227 DAVQVVDMSDS GYDVGGKYN YFKAGY NQNI SGDPDD YVQAT FYR IHKTHDQ YAADA YFKAGY NQN I SGDPDD YVQAT FYR IHKTHDQ 243 TVYEIDLSKG. VDDKDYPQ G YAKDA YFKAGY NQ GT NG ESEAHFTELSYHVKT.P YKAGY NQ FTNGESEAHFTELSYHVKT.P YKKAGY NQ FTNGESEA	222 230 211 191 208 209 213 220 213 220 264 278 318 260
AAG04556.1/1-223 BAD16656.1/1-242 USW90871.1/1-267 CAZ98266.1/1-313 CAZ95239.1/1-253 Alg1/1-264 Alg2/1-278 Alg3/1-318 Alg4/1-260 Alg5/1-231	160 KGSVYFAHE - PRKG FGDEQWYEMIGTLQPSHGNQTAAPTEP - EAGTALGETESYRI DATONKLTVTLMRE GRP 179 GKLAEAYANGSAAAYLI KDGGNDYMDLNYDQS 112 QAAPLVKLLYQLRLDQGRVQALVRERPDDGGTRAYTLMDGIPLGQPESYRIGVSRGLLSVSVNGSALEQQLDPQWAYQG 132 TNAPPLVKAVFQDGQLDMQVKQNSDGTGSDVHNYFTGIK.GDLYNMEIRVTDG VAYUTMNGDTRSVDFVGKDAGWKN 172 GSKSGNKGTEECKN AYLKLPIAPI 173 KGSVFWNYEINTKGDNSK RWDYSTAVWG YDMSVVGPTA 174 GSKSGNKGTEECKN AYLKLPIAPI AKEG 175 GSVKLKISGYVTEEQGGS YDMSVVGPTA TSYPEEP 176 GSVKLKISGYVTEEQGGS GTFSGYSLDTTYNCKLVYSGGYVELFMNGSSVFRKKMEVDDLSEN 178 TDEWSYTIEVEDKKISISVTYGGETVTKSVTIGEGDMDDDMLDDT YFKAGYNONISGDPDD YVAATFYRIHKTHDQ 243 TDEWSYTIEVEDKKSGS GYDVGGKYNYFKAGYN NONISGDPDD YVAATFYRIHKTHDQ 243 TVYEIDLSKG DIDYWRHLLS YKKAGYY NO FINGESEAHFTELSYHVKR P YVAATFYRIHKTHOQ 240 NV VDKGQRMVEK DIDYWRHLS YKKAGYY NO FINGESEAHFTELSYHVKARG G 182 DS VTHSEINKLDISYWGELES YFKAGYY NO FINGESEAHFTELSYHVKAP YFKAGY NO FINGESEAHFTELSYHVGQAID	222 230 211 191 208 209 213 220 213 220 264 278 318 260 231
AAG04556.1/1-223 BAD16556.1/1-242 USW90871.1/1-267 CA298266.1/1-313 CA295239.1/1-253 Alg1/1-264 Alg2/1-278 Alg3/1-318 Alg4/1-260 Alg5/1-231 Alg5/1-209	160 KGSVYFAHE - PRKG FGDEQWYEMIGTLOPSHGNQTAAPTEP - EAGTALGETFSYRI DATGNKLTVTLMRE GRP 179 GKLAEAYANGSAAAYLI KDGGNDYMDLNYDOS GKLAEAYANGSAAAYLI KDGGNDYMDLNYDOS 112 QAAPLVKLLYQLRLDQGRVQALVRERPDDGGTRAYTLMDGIP GOPFSYRIGVSRSGLLSVSVNGSALEQQLDPQWAYQG TNAPPLVKAVFQDGQLDMQVKQNSDGTGSDVHNYFGIK GDVGNSVGSKSUSVNGSALEQQLDPQWAYQG 12 TNAPPLVKAVFQDGQLDMQVKQNSDGTGSDVHNYFGIK GDVGNSVDFVGNGAKWN AKEG.TDKFDIYVGGTSVFVKQFVQGVMAYQG 132 TNAPPLVKAVFQDGQLDMQVKQNSDGTGSDVHNYFGIK GDVGNSVFKKKWPVDG.VAYVTMNGDTRSVDFVGNGAKWN AKEG.TDKFDIYVGG 138 KGSVFWNYE INTKGDNSK RWDYSTAVWG YDMSVVGPTA TSYPEEP EDGTALGEFSYEI NVYEGIMYLTFSSE 138 KGSVFWYTEEQGGS OTFSGYSLDTTYNCKLVYSGGYVELFMNGSSVFRKKMEVDDLSEN OTFSGYSLDTTYNCKLVYSGGYVELFMNGSSVFRKKMEVDDLSEN 138 TDEWSYT I EVEDKKI SI SVTYGGETVTKSVT I GEGDMDDDWLDDT YFKAGY NON NI SGDPDD YVAFTYRI I KHTHDQ 227 DAVVVMSDSS GYDVGGKYNYFKKAGY NON NI SGDPDD YVAATFYRI I KHTHDQ 243 TVTYE I DLSKG - VDDKDYPQ G YFKAGY NO TI SGDPDD YVAATFYRI I KHTHDQ 243 TVTYE I DLSKG - VDDKDYPQ G YFKAGY NO FI SGSCG - SGDFTVDKANGDYNSVT FSOLKLN - G 206 NKTLA I DVNGORMVEK DI DYWRHLLS YFKAGY NO FI SGSTGRVQFES	222 230 211 191 208 209 213 220 213 220 264 278 318 260 231 109
AAG04556.1/1-223 BAD16656.1/1-242 USW90871.1/1-267 CA298266.1/1-313 CA295239.1/1-253 Alg1/1-264 Alg2/1-278 Alg3/1-278 Alg3/1-278 Alg3/1-280 Alg3/1-231 Alg4/1-260 Alg5/1-209 Alg5/1-209	160 KGSVYFAHE - PRKG FGDEQWYEMI GTLOPSHGNQTAAPTEP - EAGTAL GETESYRI DATONKLTVTLMRE GRP 179 GKLAEAYANGSAAAYLI KDGGNDYMDLNYDOS GKLAEAYANGSAAAYLI KDGGNDYMDLNYDOS 112 QAAPLVKLLYQLRLDQGRVQALVRERPDDGGTRAYTLMDG IPLGQPFSYRIGVSRSGLLSVSVNGSALEQQLDPQWAYQG TNAPPLVKAVGDGQLDMQVKQNSDGTGSDVHNYTGI KLGDLYNME IRVTDG-VAYYTMNGDTRSVDFVGKDAGWKN 12 TNAPPLVKAVGDGQLDMQVKQNSDGTGSDVHNYTGI KLGDLYNME IRVTDG-VAYYTMNGDTRSVDFVGKDAGWKN AKEG.TDKFDIYVGG 132 TNAPPLVKAVGDGQLDMQVKQNSDGTGSDVHNYTGI KLGDLYNME IRVTDG-VAYYTMNGDTRSVDFVGKDAGWKN AKEG.TDKFDIYVGG 134 KGSVFWNYE INTKGDNSK RWDYSTAWG YDMSVVGPTA TSYPEEP.EDGIALGEERSYEI NVYEGIMYLTFSSE GHK 157 SGSVKLKISGYTEEQGGS QTFSGYSL DTTYNCKLVYSGGYVELFMNGSSVFRKKMEVDDLSEN YFKAGN NAANKSSGGSFT VKFTDLDVSHSD 227 DAVQVVDMSDS GYDVGGKYN YFKAGV NAN NI SGDPDD YFKAGN GCS.VSETHPWGPGCQG.TGDFTVDKANGDYNSVTFSQLKLN.G YVAAT FYR HKTHOQ 243 TVYEIDLSKG.VDDKDYPQ G YKKAGN GCS.VSETHPWGPGCQG.TGDFTVDKANGDYNSVTFSQLKLN.G YKKAGN NA FKAGN NA FTNGESALMYTYNA DSGTGRVOFFESLKYYQAAID YKKAGN NA CHTKASS YKAGY NA CHTKASS YKKAGN NA CHTKASS ACQYRGAD SGDYTQVSFYQLDLARF.G 241 SKTILI HVDGQLMVEK PI DYWSHLLS YKKAGN NA CHTKASS	222 230 211 191 208 209 213 220 264 278 318 260 231 309 249
AAG04556.1/1-223 BAD16556.1/1-223 USW90871.1/1-267 CA298266.1/1-213 CA298266.1/1-213 Alg1/1-264 Alg2/1-278 Alg2/1-278 Alg2/1-278 Alg2/1-278 Alg2/1-278 Alg2/1-280 Alg2/1-231 Alg6/1-209 Alg7/1-249 BAA83339.1/1-227	160 KGSVYFAHE - PRKG FGDEQWYEMI GTLQPSHGNQTAAPTEP - EAGTAL GETESYRI DATONKLTVTLMRE GRP 179 GKLAEAYANGSAAAYLI KDGGNDYMDLNYDOS 112 QAAPLVKLLYQLRLDQGRVQALVRERPDDGGTRAYTLMDGTPLGQPFSYRIGVSRSGLLSVSVNGSALEQQLDPQWAYQG 112 TNAPPLVKAVFQDGQLDMQVKQNSDGTGSDVHNYF FGLKLGUTYMEIRVTDG-VAYVTMNGDTRSVDFVGKDAGWKN 123 TNAPPLVKAVFQDGQLDMQVKQNSDGTGSDVHNYF FGLKLGUTYMEIRVTDG-VAYVTMNGDTRSVDFVGKDAGWKN 172 GSKSGNKGTEECKN AYLKLPIAPI 1738 KGSVFWNYEINTKGDNSK RWDYSTAVWG YDMSVVGPTA TSYPEEP 1738 KGSVFWNYEINTKGDNSK RWDYSTAVWG YDMSVVGPTA TSYPEEP 174 GSVKLKISGVTEEQGGS GTFSGYSLDTTYNCKLVYSGGYVELFMNGSSVFRKKMEVDDLSEN GTFSGYSLDTTYNCKLVYSGGYVELFMNGSSVFRKKMEVDDLSEN 175 SGSVKLKISISVTYGGETVTKSVTIGEGDMDDDMLDDT YFKAGYNONISGDPDD YVQATFYRIHKTHDQ 243 TVTYEIDLSKG GYDVGGKYNYEKAGYN NN ISGDPDD YVQATFYRIHKTHDQ 243 TVTYEIDLSKG JYKKAGYNOFTKAGYNOFTKAGYNOFTSUSTFSOLKLN G2 189 DS VTHSEINKLDISYWGELES YFKAGYNOFTKAGYNOFTSUSTFSOLKLYQQAID YFKAGYNO SOS SOBTGRVQFESLKYYQQAID 241 SKTFQVDLAHG-HYGGHDIDQG YGDDWYTKAGAN QGTMYKASSS ACQYRGAD SGDYTQVSFYQLDLARF 192 VTHSEINKLDISYWGELES YFKAGYNOFTKAGYNOFTKGSALVQFESLKYQQAID <td>222 230 211 191 208 209 213 220 264 278 318 260 231 809 249 227</td>	222 230 211 191 208 209 213 220 264 278 318 260 231 809 249 227
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Figure 4. Multiple sequence alignments of Alg1–7 and nine well-characterized alginate lyases of the PL7 family. The conserved amino acid regions are marked in the black boxes.

2.5. Detection of Activity and Properties of Alginate Lyase

The fermentation supernatant was obtained by centrifugation, the alginate lyase activity of which was 39.1 U/mL, determined by the ultraviolet absorption method. Extracellular alginate lyase was prepared from the fermentation supernatant by precipitating in saturated ammonium sulfate (80%) buffer and dialyzing in a dialysis bag. The enzyme activity of the dialysis supernatant was 320.6 U/L, which was used as a partially purified alginate lyase for enzymatic properties.

The effects of temperature and pH on the activity of the partially purified alginate lyase were investigated. As shown in Figure 5a, the alginate lyase exhibited maximum enzymatic activity at 50 °C. Nearly 90% of the highest activity was indicated at the temperature range of 30–60 °C, while above 70% detectable activity was observed at 4 °C and 70 °C. The thermostability of alginate lyase was measured at a temperature ranging from 4–80 °C (Figure 5b). The alginate lyases were relatively stable at temperatures below 50 °C; approximately 90% of the activity was maintained after incubation at less than 50 °C for 2 h. When the temperature rose to above 60 °C, the rate of decrease in activity accelerated, while it still maintained over 60% activity at 80 $^{\circ}$ C for 2 h. As shown in Figure 5c, the activity was the highest at pH 7.0, and nearly 90% of the maximum activity was maintained when the pH value was between 5.0 and 10.0. The activity was the most stable at pH 8.0; above 90% of the activity was maintained at pH 3.0–9.0 for 24 h (Figure 5d). The results manifested that the alginate lyases of Vibrio sp. HB236076 exhibited good activity under wide temperature and pH ranges and exhibited excellent thermal and pH stabilities. As shown in Figure 5e, the effects of metal ions and compounds (1 mM and 10 mM) on alginase activity were detected. At 1 mM, Fe^{2+} displayed a significant promoting effect on the enzyme activity, with 2.6 times the control group; K^+ and Mg²⁺ displayed a weak promoting effect; Zn²⁺, Mn²⁺, and SDS showed obvious inhibitory effects. At 10 mM, Fe²⁺ and Mg^{2+} greatly increased alginate lyase activity, reaching 3.5 and 1.9 times of the control group, respectively; the other tested ions (Na⁺, K⁺, Zn²⁺, and Mn²⁺) had slightly promoted effects; SDS showed an obvious inhibiting effect. Surprisingly, 1 mM and 10 mM EDTA had no effect on the enzyme activity. It might be because the activity of the alginate lyase did not necessarily depend on the metal ions, or the binding between metal ions and enzymes was too strong, making EDTA difficult to chelate these metal ions.

In general, most alginate lyases from the PL7 family exhibited activity at a neutral temperature and pH, high activity within narrow temperature and pH ranges, and instability under high/low temperatures and acidic/alkaline conditions. For example, the AlyIH from Isoptericola halotolerans maintained stability at neutral pH (7.0-8.0) and temperatures below 50 °C [40], and Alg2951 from Alteromonas portus remained stable at pH 8.0 and temperatures below 30 °C [35]. Nevertheless, some alginate lyases from the genus Vibrio showed pH-stable or thermo-stable properties. Alyw202 from Vibrio sp. W2 exhibited the highest activity at 45 $^{\circ}$ C and more than 60% of the activity in a broad pH range of 3.0 to 10.0 [41]. Aly08 from Vibrio sp. SY01 remained more than 80% of its initial activity in a wide pH range (4.0–10.0) and recovered 70.8% of its initial activity following heat shock treatment for 5 min [42]. Alyw201 from Vibrio sp. W2 performed more than 80% of activity at 25–40 $^{\circ}$ C, and more than 70% of the activity was obtained in a broad pH range of 5.0–10.0 [43]. In this article, the alginate lyase from *Vibrio* sp. HB236076 showed maximum activity at 50 °C and pH 7.0, and over 90% of the maximum enzyme activity was measured in the range of 30–60 °C and pH 5.0–10.0, respectively. Approximately 90% of the activity remained after incubation at less than 50 °C for 2 h, or pH 6.0–10.0 for 24 h. The alginase exhibited a wide range of temperature and pH activities, as well as excellent temperature and pH stability.



Figure 5. The biochemical characteristics of the extracellular alginate lyases produced by strain HB236076. (a) Effect of different temperatures on the alginate lyase activity assayed at 4–80 °C. (b) Thermal stability analysis of alginate lyase assayed at 4–80 °C for 2 h. (c) Effect of different pH values on the alginate lyase activity assayed at pH 3–10. (d) pH stability analysis of alginate lyase activity assayed at pH 3–10. (d) pH stability analysis of alginate lyase activity. * p < 0.05, *** p < 0.001. The highest activity was taken as 100% in Figure 5a–d, and the initial activity without additional substance was taken as 100% in Figure 5f. Data are shown as the means \pm standard deviation, n = 3.

2.6. Enzymatic Degradation of Sodium Alginate

The partially purified alginate lyase was used to degrade polysaccharides, while TLC and ESI-MS were used to detect AOS with low DP produced by the alginate lyase. As shown in Figure 6a, no small molecule oligosaccharide appeared when incubating for 3 h. When incubated for 6 h, trisaccharides began to appear. When incubated for 24–36 h, the AOS of disaccharides and trisaccharides appeared. The oligosaccharide content at 36 h was higher than that at 24 h, and the product incubated at 36 h was used to detect by negative-ion ESI-MS (Figure 6b). The main products of the enzymatic hydrolysates were clearly distributed in disaccharides ($[\Delta DP2 - H]^- = 351.06 \text{ m/z}$) and trisaccharides ($[\Delta DP3 - H]^- = 526.76 \text{ m/z}$). The result indicated that all the extracellular alginate lyases, Alg1–7, were endo-type alginate lyases.

Current research shows that most alginate lyases exhibit polyM-specific and endo-type degradation characteristics. The majority of endo-type alginate lyases come from PL5 and PL7 families, while most exo-type alginate lyases belong to PL15 and PL17 families [44,45]. Different endo-type lyases produce different oligosaccharides, with main products typically ranging from DP2 to DP5, while exo-type lyases generally create 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH) from alginate polymers or oligosaccharides. Moreover, some lyases can perform endo- and exo-functions at the same time, such as FlAlyC of the PL6 family [46], Alg2951 of the PL7 family [35], and Alg17B of the PL17 family [47]. Up

to now, most of the characterized alginate lyases of the PL7 family are endolytic enzymes, which release oligosaccharides with low DP 2–5 as the main products [2]. In this study, the alginate lyases from the fermentation supernatant specifically degrade alginate into disaccharides and trisaccharides, and no monosaccharides. It is speculated that all the extracellular PL7 alginate lyases (Alg1–7) with signal protein were endo-type enzymes and could be used to prepare oligosaccharides of DP2 and DP3. Alg8 and Alg9 of the PL17 family without signal protein were speculated to be exo-type enzymes, which were responsible for degrading oligosaccharides into monosaccharides for cellular utilization within the cell.



Figure 6. Degradation product analysis of alginate performed by alginate lyase from strain HB236076. (a) Degradation product analysis with TLC. Lane M, the guluronic acid sodium salt monomers, dimers, and trimers. Lane 3, 6, 24, and 36, the degradation products performed for 3, 6, 24, and 36 h, respectively. (b) Degradation product analysis with ESI-MS. The DP2 and DP3 peaks represent disaccharide and trisaccharide, respectively.

2.7. Antimicrobial Activity of Alginate Oligosaccharides

The AOS degraded by the partially purified alginate lyase from strain HB236076 were investigated for antibacterial activity against several pathogens by the agar diffusion method. Antibacterial circles were observed around the wells in plates containing *Shigella dysenteriae, Aeromonas hydrophila, Staphylococcus aureus, Streptococcus agalactiae,* and *Escherichia coli,* while the AOS were 1.91 mg/mL (Figure S2). Nevertheless, no antimicrobial activity was detected when *Pseudomonas aeruginosa* and *Vibrio alginolyticus* were tested.

Bacterial biofilms provide resistance and tolerance to host immune defense and antibiotics; therefore, disrupting biofilms is a key step in eradicating persistent bacterial infections. Alginate oligosaccharide (OligoG), consisting of 96% α-L-guluronic acid and 4% β-D-mannuronic acid isomer, possesses antibacterial and anti-biofilm properties and, thus, potentiates the activity of certain antibiotics against multidrug-resistant bacteria [48]. Many AOS have exhibited strong antibacterial activity against plant and animal pathogens. The AOS, with an average DP of 6.8, was prepared from alginate by enzymatic digestion using alginase from *Flavobacterium* sp. LXA showed significant inhibition of *Pseudomonas* aeruginosa [49]. Enzymatic AOS could also decrease Salmonella colonization and improve the intestinal barrier function and performance of chickens [50]. Owing to differences in degradation mode, molecular weight, G/M ratio, and spatial conformation of degradation products, AOS exhibit various biological activities, such as antitumor, antibacterial, neuroprotective, antioxidant, promoting cell proliferation, and regulating plant growth activity. It is necessary to conduct more detailed activity research on the AOS degraded by the alginate lyase. The purification, characterization, and activity test of the alginate lyases were carried out separately in our laboratory. In conclusion, the potential novel strain of Vibrio sp. HB236076 can be used to prepare oligosaccharides of low molecular weights with potential pharmaceutical applications.

3. Materials and Methods

3.1. Materials and Strains

Sodium alginate derived from brown seaweed was obtained from Sangon (Shanghai, China). Except for the fact that the ethanol used for purifying AOS was of chromatographic grade, all other chemicals and reagents used in this study were of analytical grade. The bacteria used as test pathogens in this study were *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus agalactiae*, *Aeromonas hydrophila*, *Vibrio alginolyticus*, and *Shigella dysenteriae*, all of which were supplied from Hainan Medical University.

3.2. Screening of Alginate-Degrading Bacteria

Sargassum samples were collected from Qionghai, Hainan, China (110°40'12" E, 19°18'36" N). A 10-gram mashed sample was diluted with 90 mL sterile aged seawater and shaken thoroughly. The suspension liquid was then serially diluted with sterile aged seawater and spread on modified 2216E agar (MA; Solarbio, Beijing, China) supplemented with 0.5% sodium alginate. The plates were monitored at 24 h intervals for 5 d at 30 °C, and colonies with different morphologies were picked up and purified. The alginate-degrading activity was preliminarily screened with the agar plate method [51]. When visible colonies were formed, 10 mL 1 M CaCl₂ solution were poured onto the plates. After incubating at room temperature for several minutes (usually 5–10 min), strain HB236076 with gelation reaction was picked and preserved at -70 °C supplemented with 20% (v/v) glycerol.

3.3. Phylogenetic Analysis Based on 16S rRNA Gene

The 16S rRNA gene sequence of strain HB236076 was amplified by PCR using two universal primers, 27F and 1492R, cloned into vector pMD 19^T (TaKaRa), then sequenced to determine the almost-complete sequence of the 16S rRNA gene [52]. The determined 16S rRNA gene sequence was compared with sequences of the closely related reference organisms at the EzBioCloud (https://www.ezbiocloud.net/identify, accessed on 15 June 2024) and NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/, accessed on 15 June 2024). Multiple sequence alignment and analysis of the corresponding sequences of the most closely related type strains were carried out using the MEGA (molecular evolutionary genetics analysis) version 11 software package [53]. Phylogenetic trees were constructed using the neighbor-joining method, and evolutionary distances were calculated with the Kimura-2-parameter model based on 1000 replicates bootstrap analysis [54]. *Grimontia hollisae* ATCC 33564^T was used as an outgroup in the phylogenetic tree.

3.4. Genome Sequencing and Annotation

Cells grown on MA agar for 24 h were harvested to extract genomic DNA using the TIANamp Bacteria DNA Kit (Qiagen, DP302) following the manufacturer's protocol. The quantity and quality of the DNA were checked by 0.8% agarose gel electrophoresis, NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) and Qubit version 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). The high-quality genome sequence was sequenced using the PacBio Sequel II system (Pacific Biosciences, Menlo Park, CA, USA) at Biomarker Technologies Co., Ltd. (Beijing, China). The de novo genome assembly was performed using Hifiasm v0.12. The clean data from Illumina Hiseq2500 (San Diego, CA, USA) was sequenced using Pilon v1.22 to generate one contig without a gap to verify the contaminant. G+C content was analyzed using the RAST server and the genome sequence [55]. Gene and protein-coding sequences were predicted with Prodigal v2.6.3 [56]. Transfer RNA (tRNA) and ribosomal RNA (rRNA) were predicted with tRNAscan-SE v2.0 [57] and Infernal v1.1.3 [58], respectively. The genome was annotated with the non-redundant protein (NR), gene ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) results for gene function prediction [59-61]. The secondary metabolite gene clusters were predicted by antiSMASH 6.0 [62].

CAZymes were annotated using HMMER software against the dbCAN database (http://bcb.unl.edu/dbCAN2, accessed on 30 July 2024) [63] and InterPro database (https:

//www.ebi.ac.uk/interpro/, accessed on 30 July 2024) [64]. The theoretical isoelectronic point (pI) and molecular weight (Mw) were predicted online (https://web.expasy.org/ compute_pi/, accessed on 30 July 2024) [65]. The signal peptide was predicted using the SingalP server (https://services.healthtech.dtu.dk/services/SignalP-6.0/, accessed on 30 July 2024) [66]. A phylogenetic tree of related protein sequences from PL7 and PL17 families was constructed using the neighbor-joining method with MEGA 11 [53]. Poly (beta-D-mannuronate) lyase (ABG42142.1) from the PL6 family was used as an outgroup in the phylogenetic tree. Multiple sequence alignment was performed among the characterized PL7 family alginate lyases using MEGA 11 and obtained using Jalview V2.10.5.

For further identification of strain HB236076, a genomic comparison was carried out using average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) with the close relative members. The ANI was calculated using the OrthoANIu algorithm by the EzGenome server (http://www.ezbiocloud.net/tools/ani, accessed on 30 July 2024) [67], and the dDDH value was estimated using the genome-to-genome distance calculator [68].

3.5. Detection of Alginate Lyase Activity and Enzymatic Properties

Strain HB236076 was propagated on the optimized liquid medium consisting of 5 g/L sodium alginate, 7.5 g/L trypsin, 25 g/L NaCl, 0.1 g/L MgSO₄ · 7H₂O, and pH 7.0 at 28 °C and 180 rpm for 12 h. Cells were removed by centrifugation at 10,000 rpm, 4 °C for 10 min, and then the alginate lyase activity of the supernatant was measured by the ultraviolet absorption method [69]. One unit of enzyme activity was defined as an increase of 0.1 in absorbance per min at 235 nm.

Unless otherwise stated, the following operating temperature of alginate lyase was carried out at 4 °C. Solid ammonium sulfate was added slowly to the cell-free crude enzyme solution to obtain 80% saturation and kept overnight. The resultant precipitate was centrifuged at 10,000 rpm for 15 min and then dissolved in 0.01 M phosphate-citrate buffer (pH 7.0). The enzyme solution was dialyzed four times in a dialysis bag (MWCO: 8000 Da) using the same buffer, changing the dialysis buffer every four hours. The dialyzed supernatant was used as partially purified alginase for the subsequent experiments.

The activities of alginate lyase at different temperatures (4 °C, 30–80 °C at 10 °C increments) and pH 7.0 were measured to investigate the effect of temperature on the enzyme activity. The enzyme was preincubated at different temperatures (4 °C, 30–80 °C) and pH 7.0 for 2 h to investigate the effect of temperature on alginate lyase stability. The enzyme activities at various pHs using Na₂HPO₄-citric acid (pH 3.0–8.0), Tris-HCl (pH 7.0–9.0), and glycine-NaOH (pH 9.0–10.0) buffers at 40 °C were measured to investigate the effect of pH value on the enzyme activity. The enzyme was preincubated in the buffers described above (pH 3.0–10.0) at 4 °C for 24 h to investigate the effect of pH value on alginate lyase stability. The highest activity mentioned above was taken as 100%. Under the optimum temperature and pH values, the enzyme activity was measured at 1 mM and 10 mM NaCl, KCl, MgCl₂, MnSO₄, ZnSO₄, FeSO₄, ethylenediamine tetraacetic acid (EDTA), and sodium dodecyl sulfate (SDS), respectively. Alginate lyase activity was measured as described above, with an untreated activity of 100%. All reactions were performed in triplicate.

3.6. Preparation and Detection of the Degradation Products of the Alginate Lyase

To investigate the degradation pattern of the alginate lyase, alginate degradation was performed with 15 g/L sodium alginate in 50 mM phosphate buffer (pH 7.0) as a substrate. The experiments were carried out under the conditions of 19.5 U/mL enzyme, pH 7.0, and 40 °C for 0–36 h. At intervals (3, 6, 24, and 36 h), aliquot samples (10 mL) were taken, boiled for 10 min to denature the enzymes, and then centrifuged at 12,000 rpm for 10 min to remove the undissolved substances.

The lysates were precipitated overnight with three times the volume of ethanol at 4 °C and then centrifugated at 12,000 rpm for 10 min. The supernatants were collected, lyophilized, and further redissolved in ultrapure water for the next tests. The TLC method

was applied to examine the AOS with different DP produced by the alginate lyase. The degradation product compositions were analyzed by the TLC method on a silica gel high-performance TLC plate (Merck, Germany) with 1-butanol/formic acid/water (4:6:1 v:v:v) as a mobile solvent. Then, the plate was visualized by heating at 110 °C for 10 min after spraying with 10% (v/v) sulfuric acid in ethanol [70]. The guluronic acid sodium salt monomer, dimer and trimer (1 mg/mL) (Qingdao Bozhi Huili Biotech) were used as standards. In the ESI-MS analysis, the supernatant (2 µL) was loop-injected into the ESI-MS instrument (Bruker Compact, Bremen, Germany) after filtering by a 0.45 µm membrane. The oligosaccharides were detected in negative-ion mode using the following settings: end plate offset, 500 V, 131 nA; capillary, 4500 V, 2505 nA; nebulizer, 0.4 bar; dry gas, 4.0 L/min; dry temperature, 220 °C.

3.7. Assays for Antimicrobial Activity

Antimicrobial activity on seven pathogenic bacteria was determined by the agar diffusion method in this study. All the bacteria were incubated overnight at 37 °C in LB medium (Solarbio) for testing. While the autoclaved LB agar (Solarbio) was cooled to around 45 °C, the overnight culture was added and mixed to a cell density of approximately 10^{6} CFU/mL. After drilling wells (8 mm in diameter) on the inoculated LB plate, 50 µL oligosaccharide solution (1.91 mg/mL) filtered through 0.45 µm membrane were added to the wells. The diameter of the inhibition zone was measured after the inoculated LB plate was incubated at 37 °C for 24 h.

4. Conclusions

In this work, an alginate lyase-excreting marine bacterium, designated HB236076, was considered to represent a potential novel species of the genus *Vibrio* based on the phylogenetic and genetic data. The isolate presented two chromosome genomes with sizes of 3,007,948 bp and 874,895 bp. The CAZymes analysis result indicated that the isolate encoded 73 CAZymes, including nine alginate lyases in PL7 and PL17 families. The extracellular alginate lyase of strain HB236076 exhibited a wide range of temperature and pH activity and stability. The AOS of sodium alginate degraded by the extracellular alginate lyase mainly contained disaccharides and trisaccharides, which showed antimicrobial activity against several pathogenic bacteria. The results indicated that *Vibrio* sp. HB236076 was a good source of pH- and thermo-stable alginate lyase and oligosaccharide preparation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/ 10.3390/md22090414/s1: Table S1: General genome features of *Vibrio* sp. HB236076; Table S2: ANI, dDDH, and 16S rRNA gene similarity values of strain HB236076 and the phylogenetically related taxa; Table S3: The polysaccharide-degrading enzymes predicted from the genome of *Vibrio* sp. HB236076; Figure S1: Gellation reaction of strain HB236076 observed on the plate covered by CaCl₂ solution; Figure S2: Antibacterial activity testing plates.

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