Isolation and Molecular Characterization of *Shewanella* sp. G5, a producer of Cold-Active β-D-Glucosidases

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Abstract

β-Glucosidase is one of the most interesting glycosidases, especially for hydrolysis of glycoconjugated precursors, in musts and wines, and the release of active aromatic compounds. A *Shewanella* sp. G5 strain was isolated from the intestinal content of benthonic organism (*Munida subrrugosa*) from different coastal areas of the Beagle Channel, Tierra del Fuego (Argentina); this marine bacteria was able to grow at a temperature range between 4 to 20 ºC using different β-glycosides substrate, such as cellobiose, as carbon sources. In this work, the *Shewanella* sp. G5 strain exhibited high β-glucosidase activity in plate at low temperature (4 and 20 ºC). Two genes that encoding different cold-active enzymes β-glucosidases were amplified and sequenced, the nucleotides sequences were submitted to the GenBank. *Shewanella* sp. G5 was molecularly characterized employing suitable phylogenetic marker for bacteria systematic; sequence fragments of 16S rDNA gene and *gyrB* gene were reported.

1. Introduction

Microbial enzymes are capable of catalyzing all the biochemical reactions occurring within an organism that render them compatible with life. They are an essential target for the adaptation of an organism to a cold environment [1, 2]. β-glucosidase (βG, EC 3.2.1.21) is a group of hydrolases widely existing in various sources such as bacteria, fungi, plant and animal tissues. It is well known that glycosidic compounds are useful products in pharmaceutical, food, cosmetic and fine chemical industries [3]. The biological plasticity of Glycosyl Hydrolases is a consequence of the variety of β-glucosidic substrates that they can hydrolyze from disaccharides such as cellobiose and lactose, phosphorylated disaccharides, to cyanogenic glycosides and others [4]. In recent years, food industry has shown increasing interest in enzymes, in particular, the enological sector has focused its attention on pectinase and glycosidase. Glycosidase promotes the release of wine aroma via a hydrolysis mechanism of the aroma's glycosidic precursors, especially terpene glycosides that are responsible for the varietals character of many grapes. βG is capable of releasing aglycone, in turn directly responsible for the increase in wine aroma. The supplementation with βG from external sources may enhance aroma release benefiting winemaking process [5, 6]. There is a potent biological activity as a result of hydrolytic activity of βG, with several uses in the field of medicine as antitumor agents and in general biomedical research [6]. In addition, βG play other important biological roles to catalyze transglycosylation reactions, in the flavor formation of fruits, wine and sweet potato by the production of monoterpene alcohols like linalool, α-terpeneol, citronellol, nerol, and geranol [7, 8]. βG is also associated with removal of bitterness from citrus fruit juices by catalyzing the hydrolysis of naringin to prunin. Biotransformations are now well established as a means for the manufacture of pharmaceuticals, fine chemicals, and food ingredients, owing to the high selectivity of enzymes and the use of mild reaction conditions [6, 9].

Cold regions have been colonized by wide diversity psychrophiles microorganisms which have developed adaptation strategies enabling them either to survive or to live in extreme environmental conditions [1]. Despite the strong effect of low temperatures on biochemical reactions these microorganisms have developed varied adaptations in the form of finely tuned structural changes to compensate for the deleterious effects of low temperature [2]. A cold-active enzyme tends to have reduced activation energy, leading to high catalytic efficiency, which may possibly be attributed to an enhanced local or overall flexibility of the structure of the protein [10]. In order to obtain βG enzymes with high catalytic efficiency at low temperature we report, in this work, the isolation and molecular characterization of psychrotolerant bacteria that exhibited a βG high level activity at low temperatures. In addition, the genes that codified two different βG were amplified and sequenced. In this study, we also examined the phylogenetic relationship of *Shewanella* sp. G5 strain on the basis of the nucleotide sequences of their 16S rDNA and *gyrB* genes.

2. Materials and methods

2.1. Samples

On July 2001 and February 2002 *Munida subrrugosa* (benthonic organism) and sea water samples were taken from different coastal areas of the Beagle Channel (55ºS; 67ºW), Tierra del Fuego (Argentina) and collected in sterilized glass bottles. The intestinal contents of *M. subrrugosa* were used for bacteria isolation.

2.2. Microorganisms and media

Microorganism isolations were carried out in liquid Luria-Bertani medium flasks with cellobiose (LBC) containing per liter in grams: yeast extract 0.5, peptone 0.5, sodium chloride 20 and cellobiose 10; and incubated at 4 and 15 ºC during one week. Subcultures were spread onto LBC agar plates and incubated at 4 and 15 ºC respectively. All colonies appearing on the plates over a

period of 1 week were characterized morphologically and streaked for isolation onto LBC plates. The isolates were tested for a number of taxonomic key characteristic using standard procedures such as the Gram reaction, cell size and morphology by phase-contrast microscopy. The selected colonies growth was evaluated on plate solid medium with cellulose, carboxymethyl cellulose (CMCase), lactose and maltose as mainly carbon source.

2.3. β-Glucosidase Assay

β-glucosidase (βG) production by *Shewanella* sp. G5 was evaluated on LBC medium plates. After 3 days of growth at 20 ºC, the plates were sprayed with Congo red (0.1%), and then washed with sodium chloride (1 M) and acetic acid (0.1 M); a clear zone around the colonies due to βG activity was observed by degradation of cellobiose [11].

βG activity was assay in LBC medium plates using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG; Sigma) [12]. Plates with LBC medium were sprayed with 1 ml of the analog substrates (*p*NPG 100 mM) and then incubated at 4 ºC at different times.

2.3.1. Specific Activity

*p*NPG was used as substrate to assay for specific βG activity. The intracellular proteins (IP) were obtained by microbial cultures disruption in French press. The IP were added to buffer solution and incubated at different temperature and pHs.

In order to detect βG activity a standard assay was carried out: an aliquot of 100 µl IP was added to 900 µl of phosphate buffer 50 mM pH 6 (PB), containing *p*NPG as the substrate at a final concentration of 100 mM in N,N-dimetilformamide (DMF). After 60 min of incubation at 37 ºC, the enzymatic reaction was stopped by adding 100 µl of NaOH 100 mM. The absorbance of released *p*-nitrophenol from reaction was read spectrophotometrically at 420 nm.

One enzyme unit was defined as the quantity of enzyme required for hydrolysis of 1 µmol min^{-1} substrate under the previous experimental conditions. Activity data were expressed as specific productivity; all analyses were effected in triplicate.

2.3.2. The effects of pH and temperature on β-Glucosidase.

Activities were examined with the IP of culture of *Shewanella*. sp. G5. The effect of pH on enzyme activity was analyzed by placing enzyme aliquots in buffer solutions of different pH values from 3 to 10 (Citric acid-phosphate 0.627 M pH 3; Sodium Acetate-Acetic acid 0.5 M pH 4; Citric acid-Sodium Citrate 1M pH 5; Potassium phosphate 0.5M pH 6; Potassium phosphate 100 mM pH 7; Potassium phosphate 100 mM pH 8, Tris-Chloride 100 mM pH 9 and Tris-Chloride 100 mM pH 10). All of the assays were performed at 37 ºC. To determine the effect of temperature on enzymatic activity, samples were assayed at different temperatures (5, 10, 15, 20, 25, 30, 37 and 40 ºC) in PB pH 6. Both effects on residual activity were measured under the standard assay condition.

2.4. DNA extraction

G5 strain was grown in liquid LBC medium for 2 days. Total genomic DNA extraction was carried out according to the technique as described previously [13].

2.4. Phylogenetic analysis of G5 strain.

2.4.1. 16S rDNA amplification

Total genomic DNA of G5 strain was used as templates for PCR amplifications. Amplifications were performed in 25 µl reaction volumes using 16S rDNA gene of universal oligonucleotide primers 27F and 1492R (Table 1), according to a protocol described previously [14, 15]**.** Amplifications reactions were carried out in an automated thermal cycler (Perkin-Elmer). PCR

products were run in 1.0 % (w/v) agarose gel electrophoresis and visualized using an Image Analyzer Gel Doc *BIO RAD.*

2.4.2. gyrB amplification

Bacterial genomic DNA *gyrB* gene of G5 strain was PCR-amplified using UP-1 and UP-2r (Table 1), degenerate primers according to a protocol described previously [16, 17]. Amplifications reactions were carried out in an automated thermal cycler (Perkin-Elmer). PCR products were run in 1.0 % (w/v) agarose gel, stained with ethidium bromide and then visualized using an Image Analyzer Gel Doc *BIO RAD*

2.4.3 Phylogenetic Analysis

The amplified 16S rDNA and *gyrB* genes fragments were purified with QIAquick (QIAGEN) and sequencing reactions were performed with the ABI Prism 3100 Genetic Analyzer System following the manufacturer's recommendations. Complete sequences were obtained using the multiple alignment of the DNA MAN version 4.03 and with the CLUSTAL_W programs (SDSC Biology WorkBench version 3.2 (http://workbench.sdsc.edu/). The sequence of 16S rDNA was submitted to the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST) database using the Basic Local Alignment Search Tool (BLAST) program; yet BLASTn and BLASTp programs were used to compare and search the identified sequences of closely related microorganisms [18, 19].

Two phylogenetic trees were constructed with the complete and partial sequences of 16S rDNA and *gyrB* genes respectively, according to the neighbor-joining (NJ) method with the DNAMAN 4.03 and Mega3 programs. For the NJ analysis, a matrix distance was calculated according to Kimura's two-parameter correction. These programs were used for maximum-parsimony and maximum-likelihood algorithm methods. A total of 5,000 bootstrapped replicate resembling data

sets were generated (for each method) and consensus trees were made. Trees files were analyzed, illustrated and generated by Mega3 program [20].

2.6. PCR amplification of β-D-Glucosidases genes and plasmid pLGβGA

2.6.1 E. coli transformation of the plasmid pLGβGA

E. coli DH5α was transformed by electroporation using a pulse of 7.5 kV cm^{-1} range for 7 ms. Plasmid DNA was isolated using the method as described previously [21].

2.6.2. Bacterial strain and plasmids

Escherichia coli DH5α was used as host strain for cloning procedures. Plasmid encoding βG activity is derived from pLGβGA. This is a pUC18-based plasmid that contains the βG gene (*bgl-A*) from *Bacillus polymyxa* inserted at the polylinker site [22]. Plasmids pLGβGA were used as positive control in *bgl* PCR reactions.

2.6.3. β-glucosidase PCR

The *bgl-A* gene was amplified by PCR using degenerate primers designed in this study based on the complete sequence of *B. polymyxa bgl-A* gene, GLU-F and GLU-R1 (Table 1) [23, 24]. PCR conditions consisted of an initial denaturalization step of 4 min at 94 ºC followed by 10 cycles of amplification comprising a denaturalization step of 1:30 min at 94 ºC, annealing at 50 ºC for 1:30 min, and extension at 72 ºC for 2 min. This was followed by 20 cycles of amplification comprising a denaturalization step of 1:30 min at 92 ºC, annealing at 55 ºC for 1:30 min, and extension at 72 ºC for 2 min. Reactions were completed with 7 min at 72 ºC followed by cooling to 4 ºC. pLGβGA plasmid was used as positive control in the PCR reactions.

A *bgl* gene was amplified by PCR using two pars of degenerate primers based on completes sequences of *bgl* genes of members *Shewanella* genus. The primers employed, designed in this study, were GLU-F-06, GLU-R-06 and F-2000-06, R-2000-06 (Table 1). Both PCR conditions

consisted of an initial denaturalization time of 4 min at 94 ºC followed by 30 cycles of amplification comprising a denaturalization step of 1 min at 94 ºC, annealing at 57 ºC for 1 min, and extension at 72 ºC for 2 min. Reactions were completed with 4 min at 72 ºC followed by cooling to 4 ºC. Amplifications reactions were carried out in an automated thermal cycler (Perkin-Elmer, model 9700). PCR products were run at 1.0 % (w/v) agarose gel electrophoresis, stained with ethidium bromide and then visualized using an Image Analyzer Gel Doc *BIO RAD*.

The *bgl* genes that encode βG were sequenced**.** The PCR products were performed using the ABI Prism 3100 Genetic Analyzer System following the manufacturer's recommendations. The sequences of two *bgl* genes were translated to proteins and then submitted to the NCBI databases using BLAST program search for identification of closely related microorganism.

Two phylogenetic trees were constructed with the partial sequences of *bgl-A* and *bgl* genes of βG, according to the neighbor-joining (NJ) method respectively. For the NJ analysis, a matrix distance was calculated according to Kimura's two-parameter correction. These programs were used for maximum-parsimony and maximum-likelihood algorithm methods. A total of 5,000 bootstrapped replicate resembling data sets were generated (for each method) and consensus trees were made. Trees files were analyzed, illustrated and generated by Mega3 program.

3. Results and Discussion

3.1. Microorganisms

The isolation program of marine microorganisms that present cold-active enzyme activity was focused mainly on βG. From a total of 224 isolations, 87 presented a high βG activity; 72% of the positive colonies were isolated from the intestinal contents of benthonic organisms. The growth of selected colonies was evaluated on agarized medium containing cellobiose, CMCase, cellulose, lactose, *p*NPG and maltose as carbon source. From 87 colonies analyzed 72 were able to use

cellobiose or *p*NPG as carbon source. G5 strain was selected since it presented a high βG activity at 4 and 20 ºC and was used to further assay of βG activity and amplification reactions of the *bgl* genes.

3.2. Morphological, physiological and biochemical characteristics of G5 strain

G5 strain is a Gram-negative and unflagelatted bacterium with a typical rod-shaped form. The strain was able to grow in different marine culture medium: R2A, marine broth 2216, medium minimum Brunner. G5 strain was able to use a variety of substrates as carbon source such as: cellulose, cellobiose, CMCase, xylan, maltose, lactose and glucose. Cellobiose was assimilated mainly and others substrates like cellulose, xylan and glucose were assimilated in minor proportion. The psychrotolerants bacteria (or psychrotrophs) can grow at 0 ºC or below but are capable of growing at a temperature as high as 30–32 ºC, with an optimum at 20 ºC [2]. The growth of G5 strain was evaluated at temperature ranges from 4, 10, 15, 20 and 30 ºC, with an optimum around 20 ºC. According to the obtained results G5 strain was classified as a psychrotolerant bacterium.

3.3. β-glucosidase activity

βG activity was detected at 4 and 20 ºC. The enzymatic activity shows a clear zone around the colonies due to enzymatic activity observed by degradation of cellobiose; which was detected in plates at 20 ºC using Congo red (Fig. 1a). In other assay yellow colonies were observed due to enzymatic activity by hydrolysis of *p*NPG and liberation of the *p*-nitrophenol; which was detected in plate at 4 ºC (Fig. 1b).

In this work we report the use of Congo red to quickly develop a screening of positive microorganisms that present cellulolitic activity. The assay provide a simple detection system

235 which is suitable for samples with minimum labour and expense, and is applicable to small sample
236 volumes and low activities [11]
237 The results reported confirm the temperature modified the activity of the enzyme because the
238 hydrolysis of *p*NPG was increased at low temperatures observed by the yellow-colour in the zone
239 around the colonies. Similar results were observed by Weberf and Fink for other glycohydrolases
240 [12].
241 *The effect of temperature and pH on* βG *activity.*
242 The effect of pH on βG revealed the presence of two defined activity pick at pH 6 and 8.
243 However, the enzyme shows 20 % of this activity at pH 5 (Fig. 2). This broader pH range could be
244 an important factor for developing industrial application of the enzyme in food processing.
245 Hongshan et *al.* [25] and Srguleng et *al.* [26] found similar results for rhamnosidases. The thermal
246 stability of βG enzyme was evaluated. At 20 ºC the relative activity was 40%; obtaining the
247 optimal activity at 37 ºC. However another peak was obtained at 25 ºC. The stability of βG
248 between 5 and 15 ºC showed high-life in comparison to other reported cold-active enzymes for
249 temperatures, but it kept the thermo sensitive character of cold-active enzymes (Fig. 2).
250 The found results could indicate that βG possesses two isoenzymes.
251 *3.4 Phylogenetic analysis of G5 strain.*
252 *3.4.1. 16S rDNA and gyrB sequence analysis*
253 The direct sequencing of 16S and or 23S rDNA molecules by PCR technology provides a
254 phylogenetic framework which serves as the backbone for modern microbial taxonomy. However
255 there is no threshold value for 16S rDNA homology for species recognition and due to the slow
256 evolution of the 16S rDNA genes, recently diverged species may not be recognizable [27]. The
257 gyrase B (*gyrB)* gene, encoding a bacterial DNA gyrase (topoisomerase type II), has been used in

phylogenetic studies in members of the genera *Pseudomonas, Acinetobacter, Vibrio* and others. Protein encoding genes have been reported to evolve much faster than rDNAs; thus a phylogenetic analysis using the *gyrB* sequences was expected to provide higher resolution in the determination of relationships [28].

The *Shewanella* genus is a member of the Order *Alteromonadales*, Family *Alteromonadaceae* within the gamma subdivision of the *Proteobacteria*. *Shewanella* species are gram-negative, motile rods, facultative anaerobic which in general are no fermentative, although the ability to ferment glucose has been reported in a few species [29, 30]. The *Shewanella putrefaciens* species plays a prominent role as a spoilage organism of fish and other food products. However, *S. putrefaciens* strains are heterogeneous, and several new species have been described using modern molecular methods [31].

Phylogenetic analyses of G5 strain based on 16S rDNA sequences revealed that the bacteria studied belonged to γ-*Proteobacteria*, clustering robustly within the *Shewanella* genus (Fig. 3.a); which was associated with species most closely relatives were *S. baltica* OS195 (99.2%), *S. baltica* W145 (99.1%), *S. baltica* X1410 (99.1%) and *S. baltica* IAM1477^{T} (98.6%). Nucleotide sequence accession number in the GenBank for the 16S rDNA gene sequence of the *Shewanella* sp. G5, Locus: (AY398666).

The phylogenetic affiliation of the G5 strain based on *gyrB* analysis was evaluated (Fig. 3.b). The phylogenetic tree was constructed by NJ and genetic distances were computed via Kimura model. As expected phylogenetic analysis grouped G5 strain in the *Shewanella* cluster, since their sequence similarity was higher than 98%. Two types strains of *S. baltica* (AB231331 and AF387353) showed a 98% *gyrB* sequence similar to G5 strain, yet clustered with a 98% and 97% with the type strain *S. putrefaciens* (AF005670 and AF005674) respectively in this model. Nucleotide sequence accession number in the GenBank for the *gyrB* gene sequence of the

Shewanella sp. G5, Locus: DQ268831. The housekeeping gene such as *gyrB* would have been useful, but the sequences of too many genes are still missing in γ-*Proteobacteria* [32].

3.5. Amplification of the two β-glucosidases (bgl) *genes.*

The degenerate primers to amplified *bgl* genes were designed in this study, based on the conserved regions present in the different *bgl* genes and amino acid sequence of the microorganisms employed from the data base (NCBI). Table 1 showed the position of annealing primers in the target genes. Two fragments of 854 bp (Fig. 4a) and 2,057 bp (Fig. 4b) were amplified using degenerate primers and genomic DNA from *Shewanella* sp. G5. The nucleotide sequences of both PCR fragments were submitted to the GenBank. BLASTp analysis based on the nucleotides sequences of two fragments revealed that the proteins studied belong to the family of Glycosyl Hydrolases, clustering within different βG enzymes [33]. Nucleotides sequences presents high identity with *bgl* gene of members of *Shewanella* genus, these strains were employed to construct a homology tree based on *bgl* gene. According to analyses such as the ones shown in the tree (Fig. 5.a), the relationship of the 854 bp from *Shewanella* sp. G5 was associated with several βG that coded *bgl-A* sequences of different microorganisms [34]. The closest relatives of *bgl-A* gene from *Shewanella* sp. G5 was with *bgl* gene of *Shewanella* genus which showed a 98% of homology with *S. baltica* OS155 ctg167. The *bgl* gene shown was according to phylogenetic analyses. The relationships of the 2,057 bp fragment from *Shewanella* sp. G5 was associated with several βG sequences of the family 3 of Glycosyl Hhydrolases of different genus; which present the closest homology with *S. baltica* OS155 and *S. baltica* OS195 (Fig. 5.b). The GenBank accession numbers of the *bgl-A* and *bgl* genes sequences of the *Shewanella* sp. G5 amplified in this work were submitted in the NCBI database, Locus: (DQ136044 and EF141823) respectively.

4. Concluding remarks

A Glycosil Hydrolase, βG, was isolated from a psycrotolerant *Shewanella* sp. G5. The cold-active βG activity of *Shewanella* sp. G5 appears to be an interesting system from an applied point of view and our results indicate that it could be useful in low temperature and a broader range pH food processing procedures. The sequence analysis of PCR products of *bgl* genes contributes to think that there could be two responsible genes for βG activity. The results obtained by molecular methods that use sequence of 16S rDNA and *gyrB* in the identification of microorganisms and their use in taxonomy allows appropriate use of molecular systematic and contributes to the analysis of natural and industrial microbial cultures.

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Table 1.

Primers	Nucleotide sequence 5`- 3`	Target gen	Accesion Number	Reference
GLU-F	ACNMTBTAYCAYTGGGAYCTN	*bgl-A* (872 - 892)	M60210	This study
GLU-R1	GCCCAYTCRAARTTRTCVAN	*bgl-A* (1721 - 1741)		
GLU-F-06	GCARAAAGTNGCGCARATKATCCA	*bgl* (297 - 320)	NZ_AAIV01000002 NZ_AAIO01000078 NC_007954	This study
GLU-R-06	RCGNGCATCTTCTTGWAC	*bgl* (2351 - 2367)		
F-2000-06	GTNATGGCMTCWTTYAAYAGY	*bgl* (986 - 1005)		This study
R-2000-06	CARTCRCCTTCRCANGYCAT	*bgl* (2480 - 2491)		
27F	AGAGTTTGATCMTGGCTCAG	16S rDNA (8 - 27)		[14, 15]
1492R	GGTTACCTTGTTACGACTT	16S rDNA (1495 - 1510)		
UP-1	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA	*gyrB*		[16, 17]
UP-2r	AGCAGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT	*gyrB*		

Abbreviations: *bgl-A:* β-glucosidase A, *bgl*: β-glucosidase, *gyrB*: gyrase B

Table 1. List of primers employed to amplified target genes and accession numbers of the microorganisms using in the design of degenerate primers.

Fig. 1. **β-Glucosidase activity assay**. **(a)** Bacteria colonies grow on LBC medium incubated at 20 ºC for 48h, and before being stained with Congo red and washed with sodium chloride and them acid acetic. Bacterial strains assayed *Shewanella* sp. G5 (numbers 2, 3, 4 and 5); *Pseudoalteromonas* sp. 48X used as negative control (numbers 1, 6 and 7). **(b)** Bacteria colonies growth at 4 ºC on LBC medium with *p*NPG as substrate. Yellow coloration (*p*-nitrophenol) is observed in a positive colony (G5 strain indicated).

Fig. 2. (■) Effect of Temperature and (□) pH on β-Glucosidase activity of *Shewanella* sp G5. The activity was measured under standard conditions of cold with *p*NPG at 420 nm. Activity relative (%) of βG was measured at 20 ºC with an enzyme activity pH optimum at 6.0. The temperature optimum was determinate at 37 ºC in phosphate buffer.

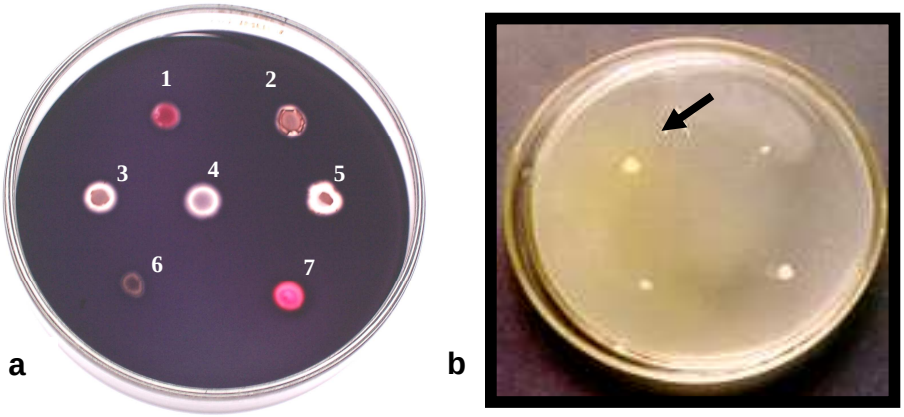
Fig. 3. Phylogenetics trees (PT) of the *Shewanella* genus. The trees were constructed by using the NJ method and genetic distances was computed by Kimura`s model. Accession numbers are given in parentheses (^{T} strains type). The scale bars indicate genetic distance and the phylogenetic position of *Shewanella* sp. G5 is indicated in bold. **a.** PT was based on 16S rDNA genes nucleotide sequences. *Pseudoalteromonas haloplanktis* and *Marinospirillum minutulum* were included as an out group. **b**. PT was based on *gyrB* gene nucleotide sequences. *P. haloplanktis* ATCC 14393 and *Alteromonas macleodii* were included as an out group.

Fig. 4. PCR amplifications of two βG genes from *Shewanella* sp. G5. Lanes **3** 1 kb DNA ladder; **1**, PCR product (854 bp) of *bglA* gene and **3**, PCR product (2057 bp) of *bgl* gene.

Fig. 5. Phylogenetic trees of β-glucosidases gene from *Shewanella* sp. G5. The trees were constructed by using the NJ method and genetic distances was computed by Kimura`s model. The scale bars indicate genetic distance and the phylogenetic position of *Shewanella* sp. G5 is

indicated in bold. Accession numbers are given in parentheses. **a**. Tree according to the *bgl-A* gene of 854 bp sequences analysis. **b.** Tree according to the *bgl* gene of 2057 bp sequences analysis.

Fig. 1



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489 **Fig. 2**

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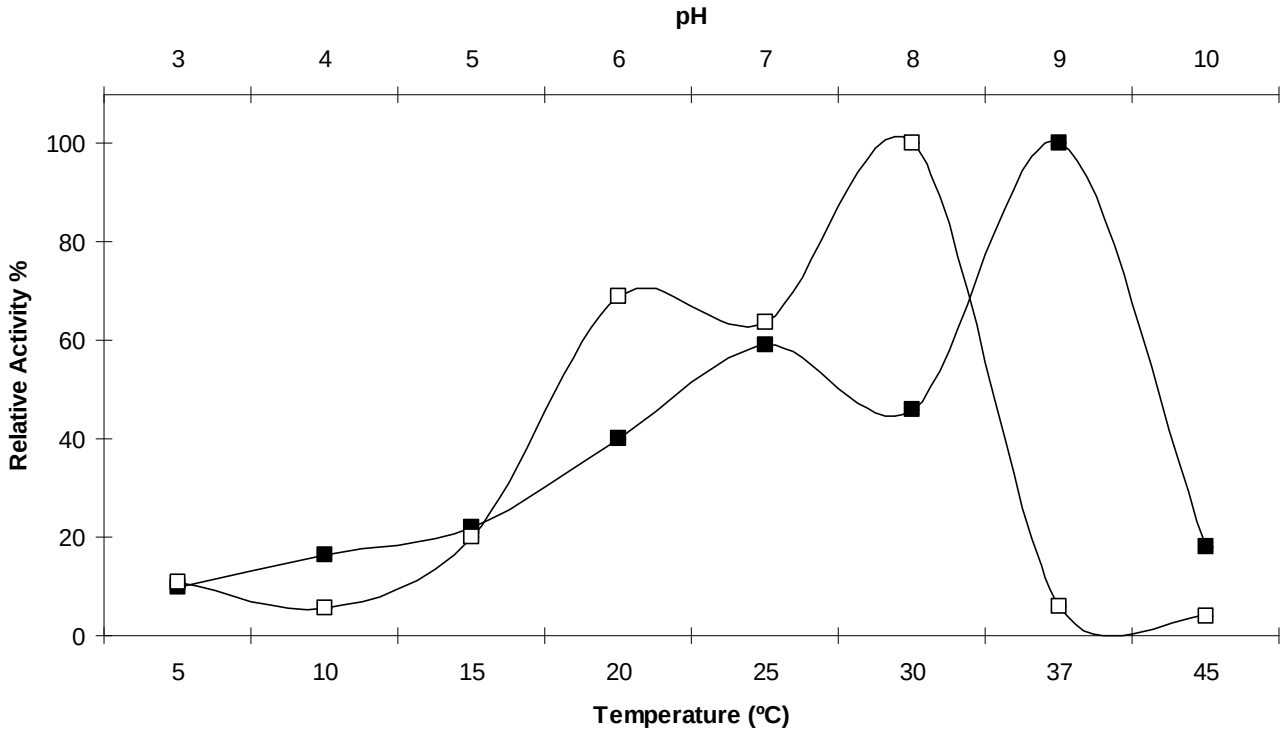

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499 **Fig. 3**

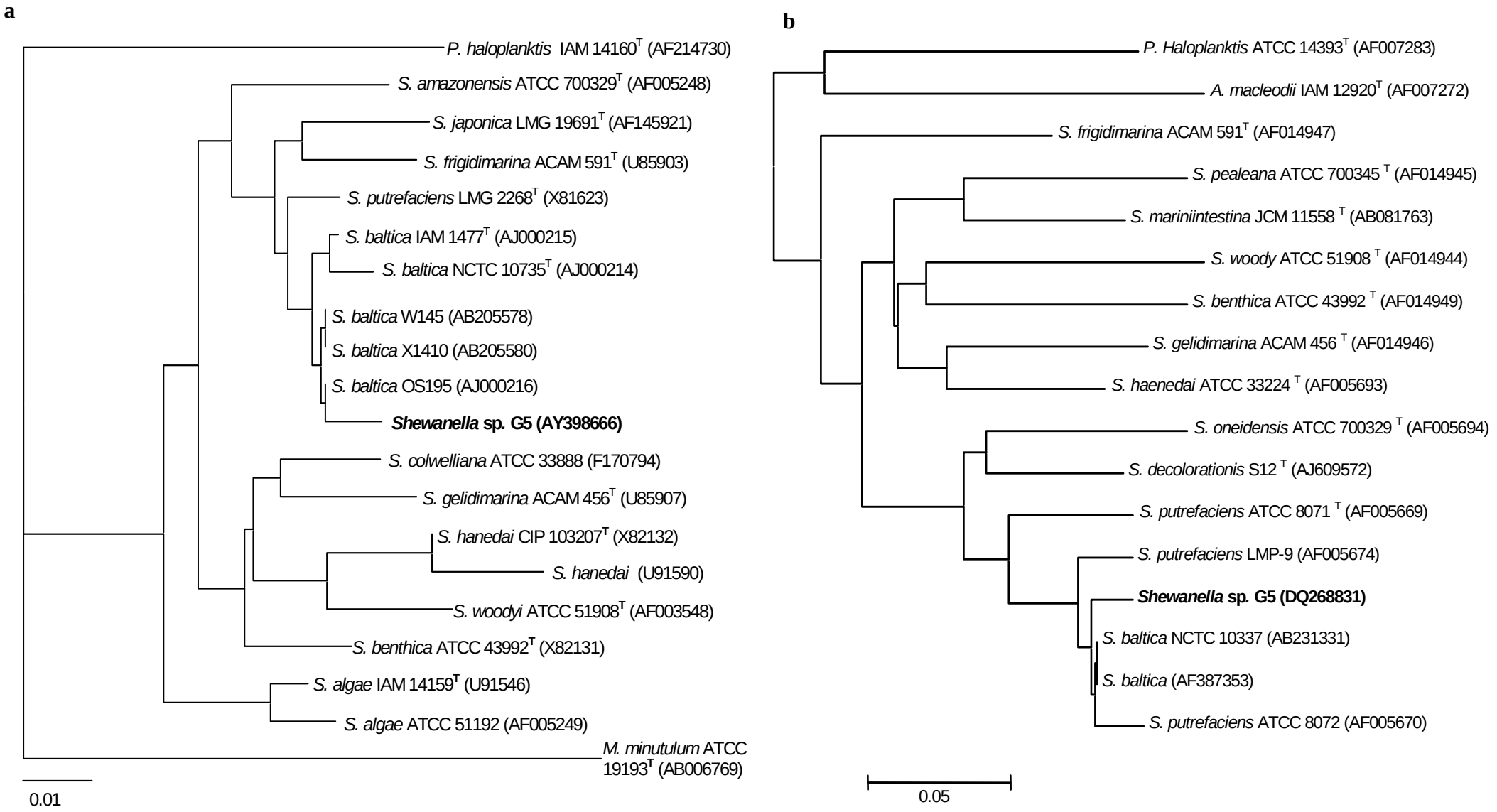

a
P. haloplanktis IAM 14160T (AF214730)
S. amazonensis ATCC 700329T (AF005248)
S. japonica LMG 19691T (AF145921)
S. frigidimarina ACAM 591T (U85903)
S. putrefaciens LMG 2268T (X81623)
S. baltica IAM 1477T (AJ000215)
S. baltica NCTC 10735T (AJ000214)
S. baltica W145 (AB205578)
S. baltica X1410 (AB205580)
S. baltica OS195 (AJ000216)
Shewanella sp. G5 (AY398666)
S. colwelliana ATCC 33888 (F170794)
S. gelidimarina ACAM 456T (U85907)
S. hanedai CIP 103207T (X82132)
S. hanedai (U91590)
S. woodyi ATCC 51908T (AF003548)
S. benthica ATCC 43992T (X82131)
S. algae IAM 14159T (U91546)
S. algae ATCC 51192 (AF005249)
M. minutulum ATCC 19193T (AB006769)
0.01
b
P. Haloplanktis ATCC 14393T (AF007283)
A. macleodii IAM 12920T (AF007272)
S. frigidimarina ACAM 591T (AF014947)
S. pealeana ATCC 700345 T (AF014945)
S. mariniintestina JCM 11558 T (AB081763)
S. woody ATCC 51908 T (AF014944)
S. benthica ATCC 43992 T (AF014949)
S. gelidimarina ACAM 456 T (AF014946)
S. haenedai ATCC 33224 T (AF005693)
S. oneidensis ATCC 700329 T (AF005694)
S. decolorationis S12 T (AJ609572)
S. putrefaciens ATCC 8071 T (AF005669)
S. putrefaciens LMP-9 (AF005674)
Shewanella sp. G5 (DQ268831)
S. baltica NCTC 10337 (AB231331)
S. baltica (AF387353)
S. putrefaciens ATCC 8072 (AF005670)
0.05

Fig. 4

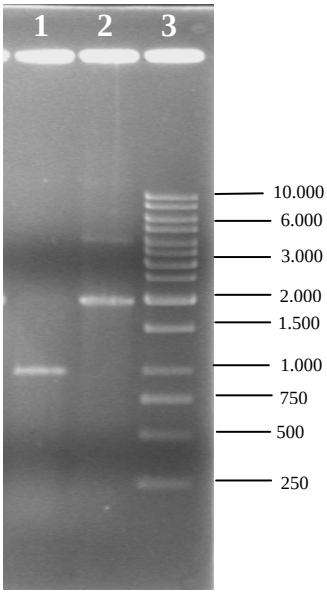
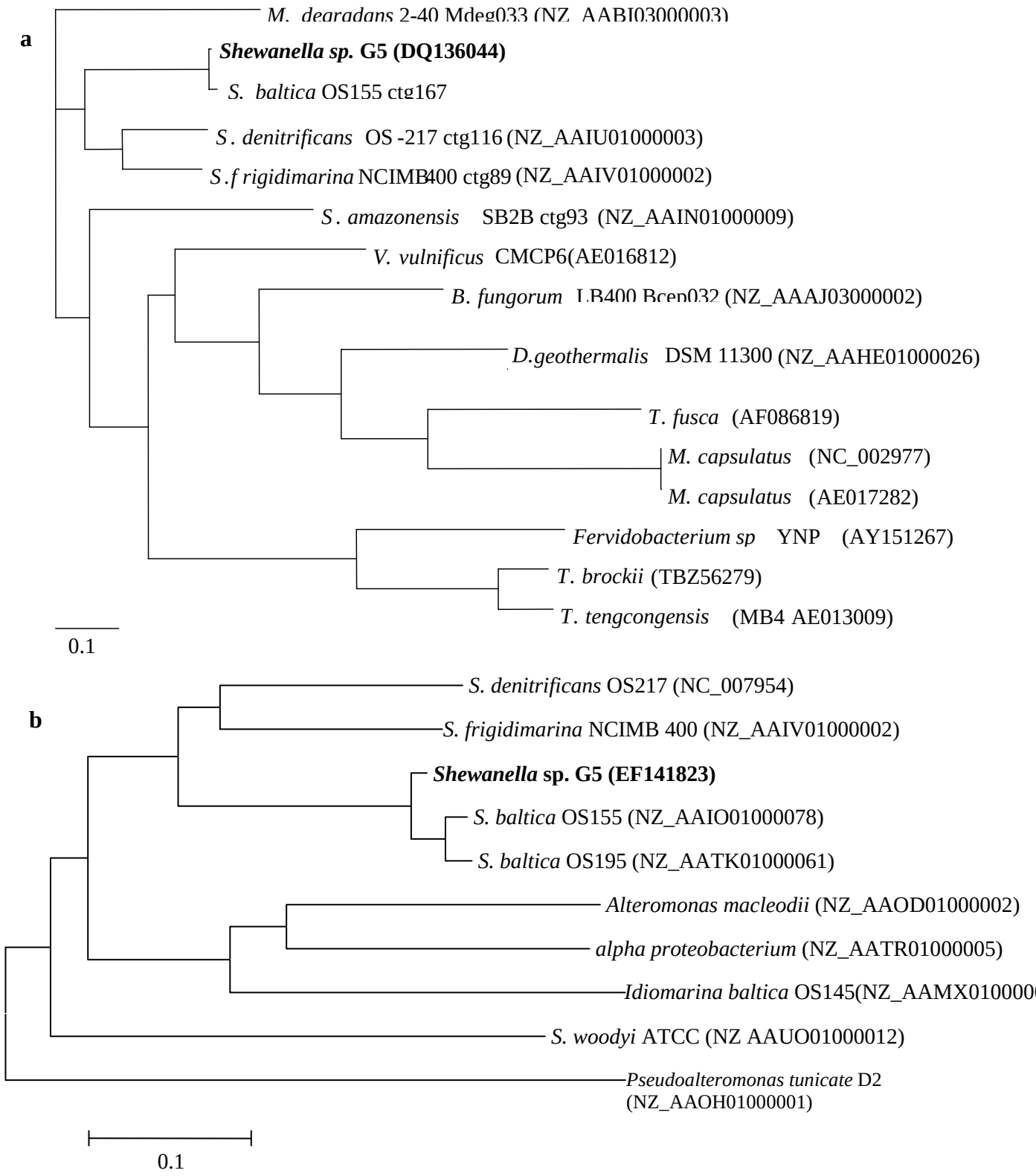


Fig. 5


a
M. degradans 2-40 Mdeg033 (NZ_AABJ03000003)
Shewanella sp. G5 (DQ136044)
S. baltica OS155 ctg167
S. denitrificans OS -217 ctg116 (NZ_AAIU01000003)
S.f rigidimarina NCIMB400 ctg89 (NZ_AAIV01000002)
S. amazonensis SB2B ctg93 (NZ_AAIN01000009)
V. vulnificus CMCP6(AE016812)
B. fungorum LB400 Bcep032 (NZ_AAAJ03000002)
D.geothermalis DSM 11300 (NZ_AAHE01000026)
T. fusca (AF086819)
M. capsulatus (NC_002977)
M. capsulatus (AE017282)
Fervidobacterium sp YNP (AY151267)
T. brockii (TBZ56279)
T. tengcongensis (MB4 AE013009)
0.1
b
S. denitrificans OS217 (NC_007954)
S. frigidimarina NCIMB 400 (NZ_AAIV01000002)
Shewanella sp. G5 (EF141823)
S. baltica OS155 (NZ_AAIO01000078)
S. baltica OS195 (NZ_AATK01000061)
Alteromonas macleodii (NZ_AAOD01000002)
alpha proteobacterium (NZ_AATR01000005)
Idiomarina baltica OS145(NZ_AAMX010000
S. woodyi ATCC (NZ AAUO01000012)
Pseudoalteromonas tunicate D2
(NZ_AAOH01000001)
0.1