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Development of Detection Method for Cyclomaltodextrinase Family Genes using Degenerate PCR Primers

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Abstract Cyclomaltodextrinases (CDases), maltogenic amylases, and neopullulanases share highly conserved primary structures and similar characteristics, and are thus classified into the same family. BLAST search has showed that a variety of bacterial strains harbor putative CDase family genes with several well-conserved motif amino acid sequences. In this study, four degenerate polymerase chain reaction (PCR) primer sets were designed for the detection of CDase genes, on the basis of their highly conserved amino acid blocks (WYQIFP, DGWRLD, LGSHDT, and KCMVW). The PCR detection conditions were optimized and the detection specificity of each for the primer sets was tested against the genomic DNAs isolated from 23 different *Bacillus*-associated species. Consequently, all tested primer sets evidenced successful amplification of specific PCR products in length, which share 55-98% amino acid sequence identity with known and putative CDases. The primers developed herein, therefore, can be applied for the easy and efficient detection and isolation of CDase family genes for the modification of functional food carbohydrates.

Keywords: degenerate PCR primers, cyclomaltodextrinases, maltogenic amylases, neopullulanases, functional food carbohydrates

Introduction

Cyclodextrin-degrading enzymes, including cyclomaltodextrinases (CDases; EC 3.2.1.54), maltogenic amylases (MAases; EC 3.2.1.133), and neopullulanases (NPases; EC 3.2.1.135) have far higher hydrolyzing activity against cyclodextrins (CDs) than against starch and pullulan, which renders them clearly differentiable from other typical amylases (1). This group of enzymes not only hydrolyzes carbohydrate substrates, but is also capable of transferring the hydrolyzed sugar moiety simultaneously to a variety of acceptor molecules via the formation of α-(1,3)-, α-(1,4)-, or α-(1,6)-glycosidic linkages. Moreover, MAases have been shown to hydrolyze acarbose, a potent glucosidase inhibitor, into glucose and acarviosine-glucose (2). As the result of their broad substrate specificity and transferring activity, the CDase-family enzymes may prove applicable to the development of functional carbohydrate derivatives in both the food and pharmaceutical industries (3-8). After the initial report of a CDase from *Bacillus macerans* (9), many CDase-like enzymes have been isolated and characterized from a variety of microbial sources, including *Thermoactinomyces* (10), *Klebsiella* (11), *Thermus* (12), *Flavobacterium* (13), *Lactobacillus* (14), but primarily *Bacillus* species (15-21). On the basis of their crystal structures and biochemical characteristics, Lee *et al.* (22) asserted that these three enzyme groups are almost indistinguishable from one another, and are sufficiently similar to be classified under the same name.

Over the past decade, an explosion has occurred in the amount of available biological information. More and more genomes are being sequenced, and valuable data regarding genes and their gene products have been accumulated. In particular, the CDase family genes are distributed widely in a variety of microorganisms, and share highly conserved primary structures among them. Presently, two major techniques are employed to detect or clone any members of a gene family on the basis of nucleotide sequence similarities. One of these techniques is colony or plaque hybridization, and the other is polymerase chain reaction (PCR) using degenerate (harboring possible mixed nucleotide bases at the same positions) primers. The former method is somewhat time-consuming and laborious, whereas the latter is relatively simple, cheap, and versatile. Degenerate PCR has proven very powerful tool in the detection of new genes or gene families (23-28). In this study, therefore, (i) several highly conserved sequence blocks on the putative CDase genes have been mined from the genome database, (ii) degenerate PCR primers have been designed and applied to the detection of CDases, (iii) the distribution of CDase family genes in nature has been characterized via experimental approaches.

Materials and Methods

Bacterial strains and growth conditions A total of 23 *Bacillus*-associated strains were purchased from the KCTC (Korean Collection for Type Cultures, Daejeon, Korea) and cultivated under optimal growth conditions and media, in accordance with the protocol established by the KCTC. *Escherichia coli* MC1061 was utilized as a host for the cloning of target genes, and the resultant transformants

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were grown in Luria-Bertani (LB) medium containing ampicillin (100 μg/mL) at 37°C.

Enzymes and reagents *Taq* DNA polymerase and restriction endonucleases were purchased from Roche Applied Science (Mannheim, Germany) or from Takara Biomedical, Inc. (Otsu, Japan). Other chemicals and reagents were purchased from the Sigma-Aldrich Co. (St. Louis, MO, USA), Merck (Darmstadt, Germany), or DUCHEFA Biochemie (Haarlem, The Netherlands). The agarose for electrophoresis and the GENECLEAN *Turbo* Nucleic Acid Purification Kit were provided by QBiogene (Carlsbad, CA, USA). The *AccuPrep* Plasmid Extraction Kit, PCR Purification Kit, and Genomic DNA Extraction Kit were obtained from the Bioneer Co. (Daejeon, Korea). Commercial ready-made T-cloning vector (pMD18-T; Takara Biomedical Inc.) was employed for the direct cloning of the PCR-amplified products. Oligonucleotide primers utilized for degenerate PCR and DNA sequencing analyses were synthesized by the Bioneer Co.

PCR amplification by using degenerate primers In order to eliminate any experimental discrepancies in the purity and quantity of the DNA templates, each genomic DNA was evenly extracted and prepared using an *AccuPrep* Genomic DNA Extraction Kit. All degenerate primers were synthesized by the Bioneer Co., and the PCR were conducted using the Px2 thermal cycler (Thermo-Hybaid, UK). The amplification of genomic DNA was carried out in 50 μL of a solution containing *Taq* polymerase buffer (10 mM Tris-HCl, pH 8.3, 40 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTPs (dATP, dGTP, dTTP, dCTP), 50-100 ng of template DNA, 0.2 pmole of each primer, and 2.5 units of *Taq* DNA polymerase (Roche Applied Science, Indianapolis, IN, USA). PCR amplification was performed in a thermal cycler as follows: an initial denaturation step for 1 min at 94°C followed by 30 cycles of 30 sec at 94°C; 30 sec at 50°C for all primer sets (determined by a temperature gradient PCR); 1 min 30 sec at 72°C, and a final cycle of 5 min at 72°C.

Cloning of PCR products PCR-amplified products were recovered using an *AccuPrep* PCR Purification Kit. For cloning, the resultant PCR products were ligated directly into pMD18-T Easy cloning vector, in accordance with the manufacturer's instructions. Ligation mixture was then transformed into $CaCl_2$-competent *E. coli* MC1061 cells via the standard heat shock protocol (29). The transformants were selected on LB-medium containing ampicillin (100 μg/mL) at 37°C. Recombinant plasmids harboring probable DNA fragments were extracted and verified via restriction endonuclease treatment and DNA sequencing analysis.

DNA sequencing and sequence analysis DNA sequencing was conducted by the Genome Research Facility of the Seoul National University (Seoul, Korea), using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. The universal sequencing primers, M13 reverse and M13 forward, were employed for the determination of the whole nucleotide sequences from the putative CDase gene fragments cloned. Sequence alignment was conducted using a local version of CLUSTAL W (30). Sequence comparisons against the Genbank, EMBL, and SwissProt databases were carried out using the BLAST (31) software available at the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) website.

Results and Discussion

Distribution of the putative CDase family genes in nature As previously mentioned, valuable genetic information is currently readily available and accessible, as the result of worldwide genome projects. According to the genome database, a variety of microorganisms harbor CDase-like genes within their genomes, and share more than 40% amino acid identity with one another. In particular, the MAase from *Thermus* strain IM6501 (ThMA; 12) is a well-characterized member of the CDase family of enzymes, and evidences a typical protein structure and enzymatic functions. As is shown in Table 1, the amino acid sequence of ThMA was aligned and compared with a variety of other sequences available at the NCBI database using the BLAST program. Interestingly, BLAST search results indicated that the CDase-family genes are extensively distributed throughout the microbial world, primarily in *Bacillus* species, and evidence a high degree of similarity with regard to their primary structures.

Despite their wide distribution in nature, the precise roles of these enzymes in bacterial cells have yet to be elucidated. Recently, the physiological roles of these unique intracellular enzymes, coupled with the findings of versatile carbohydrate transport systems in microorganisms have been the focus of increasing research efforts. Fiedler *et al.* (37) reported the existence of a novel starch degradation pathway involving the extracellular conversion of starch into CDs by cyclodextrin glycosyltransferase, the uptake of the CDs by a specific uptake system, and intracellular linearization by a CDase. In addition, Park (38) suggested that CDases might be involved in the modulation of glycogen degradation and rearrangement. In this study, we have determined the extensive distribution of CDase-like enzyme genes in the microbial world via both bioinformatics and experimental approaches. Considering their versatile transglycosylation activities in carbohydrate engineering, the degenerate PCR primers developed herein may prove applicable to the high-throughput detection and isolation of a variety of microbial CDases for industrial purposes.

Design of degenerate PCR primers In general, CDases, MAases, and NPases possess a unique additional N-terminal domain which consists of approximately 130 amino acid residues, and allows for the differentiation of these enzymes from other common amylolytic enzymes. Lee *et al.* (22) reported that the extra-long N-terminus is, therefore, likely to be an effective marker for the screening of any putative CDase gene from a large number of amylolytic enzyme genes in a database. It has been determined that the N-domain performs important functional roles in substrate-specificity and structural roles in dimer-formation (39).

Table 1. Putative CDase family genes found in microbial genome database

Microorganisms	Enzyme[1)]	Protein ID[2)]	Amino acid identity (%)[3)]	Ref
Thermus sp. IM6501	MA	AAC15072	100	12
Bacillus sp. WPD616	AMY	AAX85453	99	21
Thermus sp. YBJ-1	CD	AAL62457	96	-
Geobacillus kaustophilus HTA426	CD	BAD74988	93	-
Bacillus stearothermophilus TRS40	NP	AAA22622	86	15
Bacillus stearothermophilus IMA6503	NP	AAK15003	86	20
Bacillus thermoalkalophilus ET2	MA	AAT94159	71	-
Bacillus stearothermophilus ET1	MA	AAC46346	69	18
Anoxybacillus flavithermus	CD	AAX29991	69	32
Oceanobacillus iheyensis HTE831	CD	NP693482	63	-
Bacillus anthracis 'Ames Ancestor'	AMY	AAT33347	61	-
Bacillus thuringiensis serovar. *konkukian* 97-27	NP	AAT61258	60	-
Bacillus cereus ATCC14579	NP	AAP10933	59	-
Bacillus acidopullulyticus	MA	CAA80246	59	-
Bacillus sp. A2-5a	CD	BAA31576	58	33
Bacillus halodurans C-125	MA	BAB06646	58	-
Bacillus sp. KSM-1876	NP	BAA02521	57	34
Bacillus clausii KSM-K16	MA	BAD66562	57	-
Bacillus subtilis SUH4-2	MA	AAF23874	56	19
Bacillus licheniformis ATCC14580	MA	AAU22247	55	-
Listeria monocytogenes EGD-e	MA	NP465650	53	-
Listeria innocua Clip11262	HP	NP471546	53	-
Bacillus sphaericus E-244	CD	CAA44454	52	16
Enterococcus faecalis V583	GH	NP815068	52	35
Lactococcus lactis subsp. *lactis* Il1403	NP	NP267838	51	-
Thermoanaerobacter thermohydrosulfuricus 39E	CD	AAA23219	50	36
Streptococcus pneumoniae R6	NP	AAK99752	50	-

[1)]Enzyme classifications and abbreviations assigned by NCBI database: CD, cyclomaltodextrinase; GH, glucan hydrolase; NP, neopullulanase; MA, maltogenic amylase; AMY, amylase; HP, hypothetical protein.
[2)]Each gene product can be identified from NCBI database by using its own number.
[3)]Relative identities were determined by amino acid sequence alignment based on that of ThMA as 100%.

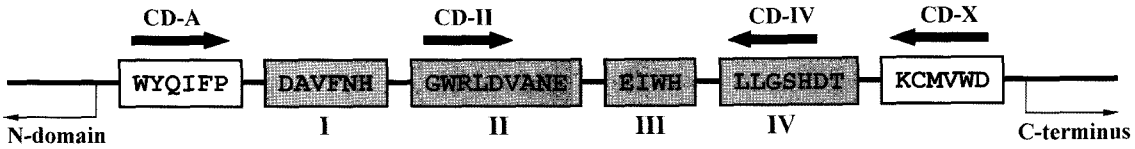

Fig. 1. Schematic representation of the location and orientation of four degenerate PCR primers on the primary structure of ThMA. Amino acid residues shown in dark gray boxes represent the common conserved regions, I, II, III, and IV, found in the majority of amylolytic enzymes. All amino acid sequences in the figure correspond to those of ThMA (12). Black arrows indicate the location and orientation of degenerate primers developed herein.

In order to determine the most suitable regions for the design of degenerate primers allowing for the amplification of CDases, highly common sequence blocks should be analyzed from a variety of putative CDase family genes. Initially, the protein sequences of the known and putative CDases were closely aligned, and compared using the CLUSTAL W program. The alignment of the CDase sequences showed that 6 amino acid blocks (WYQIFP, DAVFNH, DGWRLD, EIWH, LGSHDT, and KCMVW; shown in Fig. 1) are highly conserved throughout the majority of the sequences. As one of the principal objectives of this study was to detect only CDase family genes and not the other type of common amylolytic enzymes, such as α-amylases, the sequences in each block

were compared closely with those observed in other enzymes. Whereas the amino acid block harboring DAVFNH is frequently found in most typical amylases (40), the block including EIWH is too short for the design of any stable primer with an adequate annealing temperature. Four conserved blocks, evidencing high identity and appropriate nucleotides in length, were chosen for the design of the degenerate PCR primers. The sequences of the primers were generated from the multiple sequence alignment of the conserved nucleotide sequences among a variety of known or putative CDases (shown in Table 1). As a result, a total of four degenerate primer sets were designed for the PCR detection of the putative CDases (Table 2). Specific DNA fragments of 305, 866, 458, and 1,019 bp, respectively, were predicted to be detected when the bacterial genomic DNA template harboring a CDase gene was amplified using *Taq* polymerase coupled with degenerate primer sets #1-4.

Detection of CDase genes via degenerate PCR In order to evaluate the specificity and efficacy of the degenerate primers, 23 different *Bacillus*-associated strains were purchased from the KCTC, and the genomic DNAs evidencing appropriate purity and concentration were evenly isolated and prepared using a genomic DNA extraction kit. PCR conditions for specific detection were then optimized for template and primer concentra-tion, annealing temperature, extension time, and number of cycles. The optimized detection conditions were determined as follows: an initial denaturation step for 1 min at 94°C, followed by 30 cycles of 30 sec at 94°C; 30 sec at 50 for all primer sets; 1 min 30 sec at 72°C, and a final cycle of 5 min at 72°C.

In order to determine the specificity of the degenerate primers, a recombinant plasmid including the ThMA gene (p6xHThMA; 12) was employed as a template DNA for PCR detection. It was utilized as the positive control to obtain clear signals from PCR detection using each of the primer sets. As shown in Fig. 2A, specifically amplified PCR products with the appropriate size were detected successfully from the positive control experiments. However, the specificity or efficacy of the genomic DNA templates was dependent on the primers and microbial sources. For example, the results in Fig. 2B indicated that *Bacillus licheniformis* KCTC2107 is likely to harbor a CDase gene within its genome. As *B. licheniformis* KCTC2107 was previously reported to have a maltogenic

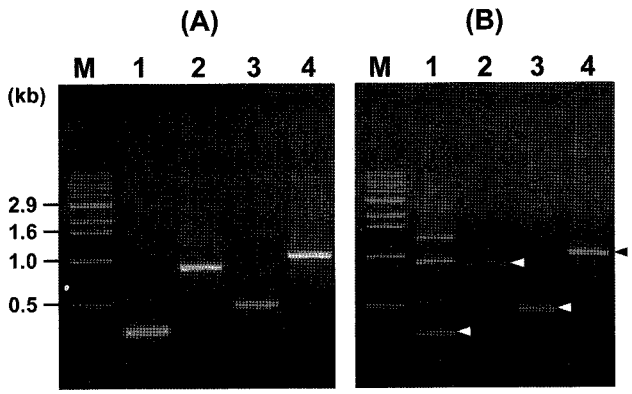

Fig. 2. PCR Detection of known CDase genes with each degenerate primer set. Panel (A) shows the results with a plasmid (p6xHThMA) harboring the ThMA gene as a template. Panel (B) corresponds to those with the genomic DNA of *B. licheniformis* KCTC1039 as a template. Specific PCR products were amplified and detected using individual primer sets: (lane M) DNA size markers (Bioneer Co., Korea); (lane 1) 305 bp fragment for primer set #1; (lane 2) 866 bp for primer set #2; (lane 3) 458 bp for primer set #3; (lane 4) 1,019 bp for primer set #4. White and black arrowheads indicate the PCR-amplified fragments with the appropriate length.

amylase gene (BLMA; 41), it can also be used as a positive control to verify primer specificity on putative CDases. Figure 3 shows PCR-amplified fragments generated via a variety of degenerate primer sets, using the genomic DNA from *Bacillus stearothermophilus* KCTC 2107 and *Geobacillus stearothermophilus* KCTC1752 as templates. Each of the fragments was detected specifically at the expected position via agarose gel electrophoresis. These results indicate that all four primer sets work quite well for the PCR-based detection of CDase-family genes. In a similar fashion, PCR detection experiments were conducted with the other 20 strains, and the PCR patterns were found to be quite similar to those of *B. stearothermophilus*. In accordance with the results summarized in Table 3, the *Bacillus* strains tested herein were expected to harbor at least one of the putative CDase genes within their chromosomal DNA. Although primer sets #2 and #4 were partially effective in this regard, primer sets #1 and #3 evidenced broad specificities for PCR detection on the putative CDase genes. In the cases of sets #1 and #3, non-specific minor bands were co-

Table 2. List of degenerate PCR primer sets for the detection of CDase family genes

Set	Primer	Primer sequence[1)]	T_m (°C)	Orientation	Degeneracy	Length (bp)
#1	CD-II	5'-GACGGYTGGCGBYTNGATGT-3'	60-68	forward	48	305
	CD-IV	5'-TCATGACTGCCSAGCARRTT-3'	58-62	reverse	8	
#2	CD-A	5'-TGGTATCAAATYTTYCCNGA-3'	52-58	forward	16	866
	CD-IV	5'-TCATGACTGCCSAGCARRTT-3'	58-62	reverse	8	
#3	CD-II	5'-GACGGYTGGCGBYTNGATGT-3'	60-68	forward	48	458
	CD-X	5'-TCCCAMACCATRCAYTT-3'	46-52	reverse	8	
#4	CD-A	5'-TGGTATCAAATYTTYCCNGA-3'	52-58	forward	16	1,019
	CD-X	5'-TCCCAMACCATRCAYTT-3'	46-52	reverse	8	

[1)]Abbreviations of degenerate nucleotides: B = G/T/C; M = A/C; N = A/C/G/T; R = A/G; S = G/C; Y = C/T.

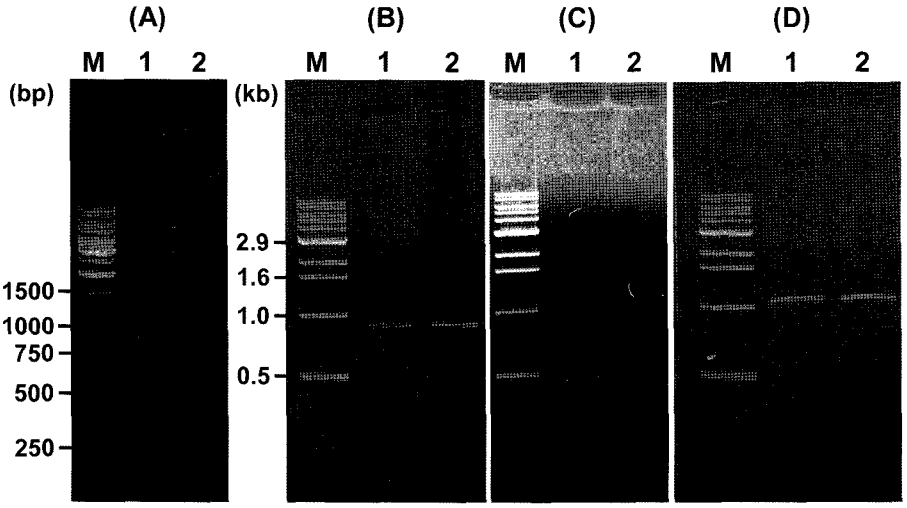

Fig. 3. Examples for the detection of CDase genes by different degenerate PCR primer sets. The efficacy and the specificity of degenerate PCR detection was determined using different primer sets and genomic DNA from *B. stearothermophilus* KCTC2107 (lane 1) and *Geobacillus stearothermophilus* KCTC1752 (lane 2). Lanes M corresponds to the 1 kb DNA marker (Bioneer Co.) or 250 bp DNA molecular marker (Takara Biomedical Inc.). Panel (A), results from primer set #1; panel (B) from primer set #2; panel (C) from primer set #3; panel (D), PCR detection results from primer set #4.

Table 3. Summarized results of the PCR detection with degenerate primer sets

Growth temp[1)]	Source of genomic DNA (KCTC/ATCC)[2)]	PCR amplification with primer set[3)]			
		#1	#2	#3	#4
20°C	*Bacillus psychrosaccharolyticus* (3399/23296)	+		+	
30°C	*Bacillus cereus* (1014/21768)	+		+	+
	Bacillus cereus (3624/14579)	+		+	+
	Bacillus firmus (3626/14575)	+		+	
	Bacillus megaterium (3007/14581)	+		+	
	Bacillus subtilis (2217/33234)	+		+	+
	Bacillus subtilis (1028/6051A)	+		+	+
	Bacillus thuringiensis (3452/10792)	+	+	+	+
	Brevibacillus brevis (3743/8246)	+		+	+
	Paenibacillus alginolyticus (3567/-)	+		+	
	Paenibacillus amylolyticus (3455/9995)	+		+	
	Paenibacillus macerans (1822/8244)	+		+	
	Paenibacillus polymyxa (3008/8523)	+		+	
	Paenibacillus larvae (3563/49843)	+		+	
	Bacillus circulans (3004/21783)	+	+	+	
37°C	*Bacillus amyloliquefaciens* (3002/23845)	+		+	
	Bacillus coagulans (3625/7050)	+		+	+
	Bacillus sp. (3009/21594)	+	+	+	+
45°C	*Bacillus licheniformis* (1030/27811)	+	+	+	+
55°C	*Aneurinibacillus thermoaerophilus* (3741/-)	+		+	
	Bacillus stearothermophilus (2107/7953)	+	+	+	+
	Geobacillus stearothermophilus (1752/12980)	+	+	+	+
	Geobacillus sp. (3012/-)	+		+	

[1)]Based on the instruction from KCTC, each microorganism was categorized by its own growth temperature.
[2)]If possible, both KCTC and ATCC numbers were co-written in a parenthesis.
[3)]Numbers of degenerate PCR primer set were identical to those in Table 2.

amplified with the target fragment (shown in Fig. 2B). Because degenerate primers include mixed or variable nucleotide bases at some positions, high degeneracy in the primer sequence can result in relatively low specificity with non-specific bands. However, primers with low degeneracy may be associated with a reduction in non-specific amplification, but may manifest problems due to narrow detection range. The degeneracy of primer CD-II is substantially higher than that of the other primers, CD-A, CD-IV, and CD-X (Table 2). Accordingly, the combination of degeneracy in primer sets may result in some differences with regard to specificity or detection efficacy (42). Although only some portions of the strains were detected by primer sets #2 or #4, both primer sets have some merit, in that they result in more specific and longer PCR products than those generated by primer sets #1 or #3. The resulting 866 or 1,019 bp fragments are capable of covering 50-60% of the length of common CDase genes

Table 4. Pairwise sequence identity between the putative CDases detected in this study

CDases[1)]	Relative amino acid (nucleotide) sequence identity (%)[2)]				
	*Bce*CD1	*Bce*CD2	*Bci*CD	*Bli*CD	*Bst*CD
ThMA	69.2 (64.9)	69.2 (64.7)	98.4 (98.8)	65.6 (64.4)	64.4 (63.4)
*Bce*CD1		93.6 (89.7)	67.6 (64.5)	58.0 (60.0)	55.2 (59.3)
*Bce*CD2			67.6 (64.5)	57.2 (60.4)	55.2 (60.4)
*Bci*CD				64.0 (64.3)	63.2 (63.4)
*Bli*CD					82.6 (78.0)

[1)]The abbreviations of putative CDases: ThMA, *Thermus* maltogenic amylase; *Bce*CD1, CDase from *Bacillus cereus* KCTC1014; *Bce* CD2, from *B. cereus* KCTC3624; *Bci*CD, from *B. circulans* KCTC 3004; *Bli*CD, from *B. licheniformis* KCTC1030; *Bst*CD, from *B. stearothermopilus* KCTC2107.
[2)]Nucleotide sequence identities are written in parentheses.

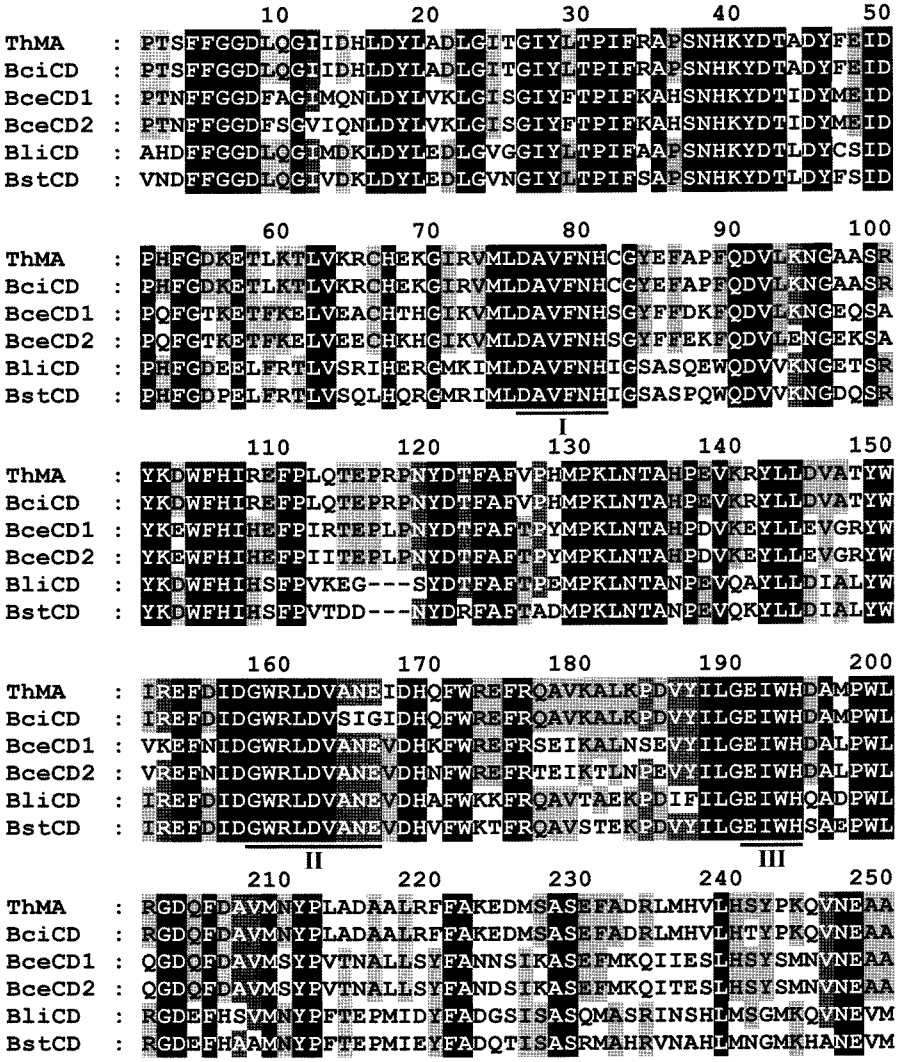

Fig. 4. Amino acid sequence alignment of ThMA and the five putative CDases detected in this study. The abbreviations of putative CDases: ThMA, *Thermus* maltogenic amylase; *Bce*CD1, CDase from *Bacillus cereus* KCTC1014; *Bce*CD2, from *B. cereus* KCTC3624; *Bci*CD, from *B. circulans* KCTC3004; *Bli*CD, from *B. licheniformis* KCTC1030; *Bst*CD, from *B. stearothermopilus* KCTC2107. Commonly known conserved amino acid sequences in most amylolytic enzymes (as shown in Fig. 1) were underlined and marked with Roman numerals I, II, and III, respectively. White letters on a black background show the fully conserved amino acid residues of all 6 proteins.

such as ThMA, which results in greater information regarding the corresponding structural genes for the purposes of gene cloning. However, primer sets #1 or #3 can be applied to the primary detection of CDase genes from a broad range of microbes. The combinational application of these primer sets may prove more useful for the acquisition of further information regarding microbial CDases.

Sequence analysis of putative CDase genes In order to characterize the sequence similarity of the PCR fragments, the fragments amplified by each of the primer sets were randomly cloned and transformed into *E. coli*. Commercial ready-made T-cloning vector (pMD18-T) was used for the high-throughput and efficient cloning of the PCR products amplified by the degenerate primers. The fragments PCR-amplified with specific primer sets #2 or #4 were expected to be 866 bp encoding 288 amino acids or 1,019 bp encoding 339 amino acids, respectively. The five probable fragments were then cloned and utilized as templates for DNA sequencing analyses. The nucleotide and deduced amino acid sequences of the detected fragments were then determined and compared with each other using CLUSTAL W software. In accordance with their nucleotide sequence alignments, five putative CDase gene fragments evidenced a broad range of amino acid sequence identity, from 55 to 98% (Table 4). The sequences of other small PCR fragments from sets #1 and #3 were simultaneously determined, revealing that they are parts of longer fragments acquired using primer sets #2 and #4 (data not shown).

In order to verify the genes in detail, all of the deduced amino acid sequences were aligned with one another, and the results were shown in Fig. 4. Common conserved regions I, II, and III were apparent in the sequence alignment of the genes. This result suggests that these may be CDase genes, and all degenerate primer sets work quite well for the detection of CDase family genes from identified bacterial strains. Actually, degenerate primer systems tend to exhibit the common problem of limited degeneracy. However, all the primers designed herein evidence a reasonable range of degeneracy and a high specificity with regard to PCR detection. Finally, the tools developed in this study will be applicable for the simple and fast detection and isolation of CDase genes from microbial sources.

Acknowledgments

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