Molecular Cloning, Overproduction and Characterization of the *Bacillus cereus* IMP Dehydrogenase

Sam-In Kim, Takahisa Miyamoto†, Ken-ichi Honjo, Masayoshi Iio, and Shoji Hatano*

Laboratory of Food Hygienic Chemistry, Division of Bioresource and Bioenvironmental Sciences, Graduate School, Kyushu University, Fukuoka 812-8581, Japan

*Nishi-Kyushu University, Saga 842-8585, Japan

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The gene of IMP dehydrogenase of *Bacillus cereus* ts-4, a temperature-sensitive mutant of *B. cereus* JCM 2152, was subcloned and its sequence was analyzed. A *B. cereus* ts-4 DNA fragment of 2,065 bp containing the entire *impdh* gene and flanking regions was sequenced. The fragment contained an open reading frame of 1,527 bp encoding 509 amino acids with a calculated molecular mass of 55,390 Da. The *impdh* sequence of JCM 2152 was also analyzed by TA cloning using PCR products amplified with primers from *B. cereus* ts-4 *impdh* gene. The gene amplified by PCR was expressed in *Escherichia coli* using a pET17 × b expression plasmid. The N-terminal amino acid sequence of the overproduced enzyme was identified as Met-Trp-Glu-Ser-Lys-Phe-Val-Lys-Glu-Gly-Leu-Thr-Phe-Asp-Asp-Leu-Val-Pro. The overproduced enzyme was eluted at a molecular mass of about 225 kDa by gel filtration. The molecular mass of the subunit was estimated to be 56 kDa by SDS-PAGE. The overproduced enzyme was active against IMP, IDP, and ITP, and showed the highest activity at pH 9.5. These properties of the recombinant enzyme were almost identical to those of IMP dehydrogenase of *B. cereus*.

**Key words:** *Bacillus cereus*; IMP dehydrogenase; sporulation; cloning; expression

**Introduction**

Depletion or limitation of nutrients, such as carbon, nitrogen, and phosphate sources, causes *Bacillus* species to sporulate. A partial decrease of the intracellular concentrations of guanine nucleotides is considered to be one of the major signals for initiation of sporulation in *B. subtilis*. IMP dehydrogenase (IMPDH) is an important enzyme involved in guanine nucleotide synthesis. It has been reported that the activity rapidly decreased when *B. subtilis* cells were transferred into starvation medium, which leads to sporulation. However, the entire mechanism of the contribution of the enzyme to induction of sporulation remains obscure.

IMPDH catalyzes the oxidation of IMP to XMP with conversion of NAD to NADH. This enzyme has been purified from several organisms and characterized. It has been reported that IMPDH was inhibited by guanosine 5'-phosphate (GMP), a competitive inhibitor with respect to IMP in *E. coli*. Similarly, Wu et al. have purified IMPDH from *B. subtilis* and shown that this enzyme was stimulated by appropriate concentrations of adenosine 5'-phosphate and inhibited by GMP. This mechanisms for regulating IMPDH activity and negative correlation between the activity and the GTP/ATP pools have also been proposed. Changes in IMPDH activity caused by the nutrient exhaustion and the nutritional shift-down have been examined in *B. subtilis* cells induced to sporulate. The enzyme activity disappears by the time of appearance of refractive spores.

In *B. cereus*, however, we have shown that IMPDH activity was greatest in the synchronous cells that had been induced to sporulate by a nutritional shift down at 40 min after the initiation of chromosome replication, the sensitive stage for sporulation. The enzyme was identified as a DNA-binding protein detected from the cells induced to sporulate. These results suggest that IMPDH of *B. cereus* ts-4 participates in the early part of sporulation.

In this paper, to obtain sufficient quantities of IMPDH for investigating function of the protein in early stage of sporulation, we have tried to clone the *impdh* gene of *B. cereus*, to overproduce *B. cereus* IMPDH in *E. coli*, and to characterize the overproduced enzyme.

**Materials and Methods**

*Bacterial strains, phages, plasmids, and culture. Bacillus cereus* ts-4 is a temperature-sensitive mutant for the initiation of chromosome replication der-

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† To whom correspondence should be addressed. Takahisa Miyamoto, Phone: +81-92-642-3024; Fax: +81-92-642-3025; E-mail: tmiyamoto@agr.kyushu-u.ac.jp
ived from B. cereus JCM 2152, which have been obtained from Japan Collection of Microorganisms, Wako, Saitama, Japan. Other strains, bacteriophages, and plasmids used are listed in Table 1. Escherichia coli strains and B. cereus strains were cultured at 37°C in LB broth. Plasmid pUC119 (Boehringer, Mannheim, Germany) was used to subclone some regions of impdh gene.

PCR amplification conditions and probe synthesis. The synthetic oligonucleotide primers 5’-CCCAT-ACACGAACGAAGGCC-3’ and 5’-ATGTGGGAATCTAATTGTTA-3’ were designed on the basis of the B. subtilis gusA gene\(^1\) and N-terminal amino acid sequence (MWESKFV) of B. cereus ts-4 IMPDH,\(^2\) respectively. A PCR product of about 1.4 kb was amplified from genomic DNA of B. cereus ts-4 by using the primer pair. PCR was done in a 50-\(\mu\)l reaction mixture containing 100 ng of template DNA, 1 × Ultra Taq buffer (Sawady Technology, Tokyo, Japan), 200 \(\mu\)M of deoxynucleotide triphosphates, 100 pmole of each forward and reverse primer, and 2 U of Ultra Taq DNA polymerase. To amplify the partial fragment of impdh gene, PCR was done for 35 cycles using B. cereus ts-4 genomic DNA as a template. PCR conditions were, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. The amplified PCR product was purified from agarose gel slices by using Gene clean II kit (BIO 101, Inc., CA, USA). The purified PCR product was labeled with digoxigenin-11-DUTP by the random primed DNA labeling method as recommended by the supplier (Boehringer, Mannheim, Germany) and used as a probe for detecting B. cereus impdh gene.

Cloning of gene encoding IMPDH of B. cereus ts-4. Two B. cereus ts-4 genomic libraries, a λ EMBL3 library of random 15- to 24-kb Sau3AI partial digests of B. cereus ts-4 genomic DNA and a λ BlueSTAR library of random 7- to 20-kb DNA of the same digests were constructed as recommended by the suppliers.

pUC119 vector was digested with BamHI and dephosphorylated by calf intestinal alkaline phosphatase (Takara, Co., Ltd., Japan). Phage DNA was purified from a positive clone obtained from λ EMBL genomic library. The phage DNA was digested with Sau3AI, and precipitated with 99% ethanol, and resuspended with TE buffer (10 mM Tris-HCl and 1 mM EDTA at pH 8.0). Ligation of Sau3AI-digested phage DNA and pUC119 treated with BamHI was done at room temperature for 2 h using T4 DNA ligase (Promega, USA). The recombinant plasmids were introduced into E. coli JM109 cells.

A positive clone obtained from a λ BlueSTAR library was subcloned automatically into pBlueSTAR by plating on a strain expressing cre recombinase, E. coli BM 25.8, in the presence of ampicillin (150 \(\mu\)g/ml). Plasmids containing inserts of interest were isolated and introduced into E. coli JM109 cells.

TA cloning of PCR products from B. cereus JCM 2152. For cloning of gene encoding IMPDH of B. cereus JCM 2152, primers were designed from the DNA sequence of B. cereus ts-4 impdh gene (Fig. 3). With these primers and Pfu DNA polymerase (Stratagene, CA, USA), several PCR products of in-

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**Table 1. Bacterial Strains, Phages, and Plasmids Used in This Study**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>Source of reference</th>
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<tr>
<td>E. coli strains</td>
<td></td>
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<tr>
<td>JM109</td>
<td>e14 (mcRA) recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δ(lac-proAB) [F (traD36 proAB lacZ Δ3 M15)]</td>
<td>29</td>
</tr>
<tr>
<td>LE392</td>
<td>F' e14 (mcRA) hsdR514 (rK12 mK12) supE44 supF58 lacY1 or Δ(lacZY)Δg2 k12 T22 metB1 trp55</td>
<td>30</td>
</tr>
<tr>
<td>BM25.8</td>
<td>supE thi Δ(lac-proAB) [F' (traD36 proA) B+ lacZY ΔM155 λ imm413 (kan8)P1(Cm8) hsdR(rK12 mK12) ]</td>
<td>31</td>
</tr>
<tr>
<td>BL21(DE3) Plasmids and phages</td>
<td>F' ompT hsdSB(rK12 mK12) gal dcm Δ(srl-recA)306:: Tn 10(Tc8)(DE3) pLysS(Cm8)</td>
<td>19</td>
</tr>
<tr>
<td>pUC119</td>
<td>Plasmid cloning vector</td>
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<tr>
<td>λ EMBL3</td>
<td>Lamda vector for genomic library</td>
<td>Promega</td>
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<tr>
<td>λ BlueSTAR</td>
<td>Lamda vector for genomic library</td>
<td>Novagen</td>
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<tr>
<td>pET17×b</td>
<td>Plasmid vector for expression</td>
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<td>GR-1</td>
<td>λ EMBL3 vector harboring Sau3AI-digested partial impdh gene</td>
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<td>pVR-1</td>
<td>pBlueSTAR vector harboring Sau3AI-digested partial impdh gene</td>
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<td>pUC119 vector harboring 236-bp Sau3AI fragment</td>
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<td>pKSI-679</td>
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<td>pGEM T-easy vector harboring 386-bp-PCR product</td>
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<tr>
<td>pET-impdh</td>
<td>pET17×b expression vector harboring ORF-PCR product of impdh gene</td>
<td>This work</td>
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terest were amplified from genomic DNA of *B. cereus* JCM 2152. The PCR mixture contained 100 ng of *B. cereus* JCM 2152 genomic DNA, 200 μM of deoxynucleotide triphosphates, 100 pmol each of the forward and reverse primers, and 2 U of *Pfu* DNA polymerase in a total volume of 50 μl. The reaction mixtures were denatured for 30 sec at 98°C, followed by 30 cycles of 30 sec at 96°C, 30 sec at 55°C, and 60 or 30 sec at 72°C and a final extension of 10 min at 72°C. Since *Pfu* DNA polymerase generates blunt-ended PCR products, it is required to add a single deoxynucleosine to the 3’ ends of the amplified products for TA cloning. For this purpose, the PCR products (3 μg) purified by the standard methods were incubated with 5 μM dATP and 5 units of Ultra Tag DNA polymerase (Sawady Technology Co. Ltd., Tokyo, Japan) at 75°C for 2 h. The PCR products (1 μg) tailed with a deoxynucleosine were ligated with pGEM-T Easy Vector (Promega, USA), which has been linearized and contains a 3’ terminal deoxythymidine on both ends, and introduced into *E. coli* JM 109.

**DNA sequencing and computer analysis.** The nucleotide sequences of the IMPDH gene were analyzed by the dideoxy chain termination method of Sanger et al. using dye-primers (Toyobo, Co. Ltd., Osaka, Japan), an AutoCycle Sequencing kit and ALExpress DNA Sequencer (Pharmacia Biotech., Uppsala, Sweden), and using dye-terminator (Amer sham Pharmacia biotech., USA), Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit and GeneRapid Sequencer system (Seq. 4 × 4) (Amersham Pharmacia biotech., USA). Sequence analysis was done using the BLAST program.

**Construction of pET-impdh and transformation of E. coli BL21 (DE3).** The T7 promoter overexpression system of *E. coli* was used for IMPDH production. A DNA fragment containing the structural gene of IMPDH of *B. cereus* ts-4 was amplified by PCR using *Pfu* DNA polymerase. The synthetic oligonucleotides 5’-GAGGAAACCATTATGTTGGGAATCTA-3’ and 5’-CCTTGGGATCCATACCCGTTATCTAAC-3’ were used to amplify IMPDH structural gene. PCR was performed for 30 cycles with 1 μg of *B. cereus* ts-4 genomic DNA. Each cycle was run at 96°C for 45 sec (denaturation), 50°C for 45 sec (annealing), and 72°C for 2 min (extension). The PCR product was digested with *NdeI* and *BamHI* and ligated into the pET 17×b (Novagen Inc., Madison, WI, USA). Plasmid harboring the *impdh* gene inserted downstream of the T7 promoter was selected and named pET-impdh. This recombinant plasmid (pET-impdh) was transferred into *E. coli* BL21 (DE3) cells.

**Overproduction and purification of the enzyme.** *E. coli* BL21 (DE3) cells carrying pET-impdh were grown in LB medium containing ampicillin (150 μg/ml) at 37°C. When the *OD* reached 0.4, IPTG was added to the culture to a final concentration of 0.5 mM. After a 12-hr incubation at 24°C, the cells were collected by centrifugation at 2500 × g for 10 min. IMPDH was purified as described previously except that the cells were disrupted by sonic treatment using a Tomy Ultrasonic Disruptor UP-201 for 10 min at 30 W with 0.5-sec pulses and 0.5-sec intervals.

**Measurement of enzyme activity.** IMPDH activity was measured by the method of Gilbert et al. One unit of enzyme activity is defined as the amount of enzyme that causes an increase of 0.001 in absorbance at 290 nm per min. Specific activity is expressed as the enzyme activity per mg protein. Proteins were measured by the method of Bradford.

**Electrophoresis and activity staining.** Enzyme samples from different purification steps were run on SDS-PAGE. Gels were stained with Coomassie blue.

Native-PAGE using 5% gel was done for activity staining. Gels were first run in Tris-glycine buffer (40 mM Tris, 190 mM glycine, and 1 mM EDTA at pH 8.5) at 100 V for at least 30 min. Samples were put on the gel immediately after this first run and run for 2 h at 100 V. After electrophoresis, the gel was incubated for 15 min at 37°C in 100 ml of a freshly prepared 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM KCl, 1 mM GSH, 1.25 mM NAD, 1.5 mM IMP, 0.55 mM nitroblue tetrazolium, and 0.16 mM phenazine methosulfate at room temperature for activity staining.

**Measurement of molecular mass.** The molecular mass of the purified enzyme was estimated by gel filtration on a TSK-Gel G3000SW column (7.7 × 300 mm, Tosoh Corp., Tokyo, Japan) and SDS-PAGE. Molecular mass standards for gel filtration were as follows: ferritin from horse spleen (450 kDa), catalase from bovine (240 kDa), and aldolase from rabbit muscle (160 kDa). For SDS-PAGE, Prestained Protein Marker, Broad Range (NEB, Biolabs, USA) was used.

**N-terminal amino acid analysis.** The N-terminal amino acid sequence of the purified enzyme was analyzed as described in our previous paper.

**Optimum pH of overproduced IMPDH.** The optimum pH of overproduced IMP dehydrogenase was examined in buffers of various pH’s. The buffers used were 50 mM Tris-acetate from pH 5.0 to 7.0, 50 mM Tris-HCl from 7.5 to 9.0, and 50 mM glycine-NaOH from 9.5 to 12.
Results and Discussion

Cloning of impdh gene

By using primers designed on the basis of the N-terminal amino acid sequence of B. cereum ts-4 IMPDH\(^{13}\) and the DNA sequence of guaB encoding IMPDH of B. subtilis,\(^{13}\) a PCR product of about 1.4 kb was amplified from B. cereum ts-4 genomic DNA as a template. This PCR product was labeled with DIG and used as a probe to screen for the impdh gene. Parts of the impdh gene were cloned from two B. cereum ts-4 genomic libraries constructed with \(\lambda\) EMBL3 and \(\lambda\) BlueSTAR. A recombinant plasmid DNA containing a partial impdh gene including start codon (ATG) and its upstream region was named GR-1. This GR-1 plasmid DNA was digested with Sau3AI and subcloned into pUC119 BamHI site to produce a pKSI-236 having a 236-bp-Sau3AI fragment and a pKSI-679 having a 679-bp-Sau3AI fragment. However, the GR-1 lacked the DNA sequence encoding the C-terminal region of IMPDH. To fill in the lacking DNA sequence, we obtained a recombinant plasmid DNA, pVR-1 containing its corresponding region of the impdh gene and the downstream region, from \(\lambda\) BlueSTAR library.

With B. cereum JCM 2152 genomic DNA and oligonucleotide primers designed from DNA sequence of B. cereum ts-4 IMPDH, PCR products with about 350 bp to 1.4 kb were obtained (Fig. 2). These PCR products were cloned into the plasmid pGEM-T easy vector to generate 5 recombinant plasmids with different inserts (Table 1).

DNA sequence of IMPDH

A B. cereum ts-4 DNA fragment with 2,065 bp that contained the IMPDH structural gene and flanking regions was sequenced (Fig. 3). The sequence has been submitted to DDBJ/EMBL/GenBank under the accession number AB035643. The impdh gene of B. cereum JCM 2152, wild-type strain, was amplified by PCR using Pfu DNA polymerase and sequenced. The sequence has also been submitted to DDBJ/EMBL/GenBank under the accession number AB036795. Nucleotide and deduced amino acid sequences of IMPDH of wild-type and ts-4 mutant B. cereum were almost identical (data not shown). Both wild-type and mutant B. cereum genes coding IMPDH contain an open reading frame of 1,527 bp encoding 509 amino acid residues with a calculated molecular mass of 55,192 Da and 55,390 Da, respectively. The deduced amino acid sequence of IMPDH was compared with entries in the GenBank and SWISS-PROT databases. A computer search of these protein databases showed that the sequence of B. cereum IMPDH has identity with deduced amino acid sequences of B. subtilis guaB (79%),\(^{13}\) Streptococcus pyogenes guaB (69%),\(^{22}\) and E. coli guaB (56%).\(^{20}\) In the region of DNA upstream of the ATG codon, sequences related to the -35 and -10 consensus promoter regions were identified by using MacVector Sequence Analysis Software (Oxford Molecular Ltd., England). The sequence, TTGACA, around -35 region of the B. cereum impdh gene, coincides with the consensus sequence. The molecular mass calculated from the deduced amino acid sequence was in good agreement with that of the native IMPDH from B. cereum ts-4.\(^{4}\) The first seven amino acid residues at N-terminus of the deduced amino acid sequence, Met-Trp-Glu-Ser-Lys-Phe-Val, also matched those of the native enzyme from B. cereum ts-4\(^{4}\) and 52-kDa DNA-binding protein.\(^{12}\)

Overproduction and purification of B. cereum IMPDH

In a previous paper,\(^{12}\) we detected IMP dehydrogenase as a DNA-binding protein from B. cereum ts-4. Therefore, we tried to overproduce B. cereum ts-4 IMPDH for investigating the function of

Fig. 1. Restriction Map of Gene Encoding B. cereum ts-4 IMPDH and DNA Subclones of the Region Carried by the Plasmids and Phages Described in Table 1.

Shaded boxes indicate the regions determined with genomic walking using PCR primers and dye-terminator. The PCR primers were designed with the end regions sequences of partial impdh gene of B. cereum ts-4 determined using pKSI-236 and pKSI-679. The direction of translation is from left to right. The translation start site (+1) is represented by the arrow. Numbering is relative to the translation start site. Restriction site abbreviations: E, EcoRI; H, HindIII; P, PstI; S, Sau3AI.

Fig. 2. Clones Used to Determine impdh Gene of B. cereum JCM 2152.

PCR was done as described in Materials and Methods, and 5 recombinant plasmids with different sizes were named as pTA-101, pTA-201, pTA-301, pTA-401, and pTA-501. The direction of translation is from left to right. The translation start site (+1) is represented by the arrow.
the protein in sporulation. The *impdh* gene was expressed from the bacteriophage T7 polymerase promoter of a pET17 × b expression vector. Cells carrying pET- *impdh* incubated at 37°C were induced by adding 0.5 mM IPTG, and incubated for another 3 h at 24°C. The cells harvested were disrupted by sonic treatment, and solvent and insoluble fractions were separated by centrifugation. Figure 4A shows the SDS-PAGE profile of whole cell proteins before and after induction and solvent and insoluble fractions of the induced cells. The overproduced enzyme of interest existed in the soluble fraction. The enzyme was purified by (NH₄)₂SO₄ precipitation and column chromatographies. The purified enzyme was mixed as a single band on a SDS-gel at a molecular mass of about 56 kDa (Fig. 4A, lane 6).

It has been reported that induction at temperature lower than growth temperature would lead to soluble and active protein. Takagi et al. have reported that a 16-fold increase in production of active subtilisin E in *E. coli* was obtained at a lower temperature (23°C). In our experiments, maximal expression of enzyme was also reached when the 37°C-grown cells were induced with 0.5 mM IPTG at 24°C.

Some properties of the overproduced enzyme

After purification of overproduced enzyme, some properties were investigated to show the authenticity of the overproduced enzyme as reported in our previous paper.  The N-terminal amino acid sequence was Met-Thr-Glu-Ser-Lys-Phe-Val-Lys-Glu-Gly-Leu-Thr-Phe-Asp-Asp-Val-Leu-Leu-Val-Pro. The first 20 residues of N-terminal amino acids of the overproduced enzyme and those of *B. cereus* ts-4 IMPDH were identical (Fig. 3).

The molecular mass of the enzyme was estimated by gel filtration on a TSK-Gel G3000SW̄ column. The overproduced enzyme was eluted at a molecular mass of about 225 kDa (Fig. 5). The molecular mass of the subunit was estimated to be about 56 kDa by SDS-PAGE, and only a single protein band was detected by activity staining (Fig. 4C). From these results, the enzyme overproduced in *E. coli* seems to be composed of four identical subunits.
of 56 kDa. The optimum pH of the enzyme was between pH9 and 10 (data not shown). The enzyme showed a high specific activity for inosine nucleotides, although it did not act on other purines and pyrimidines including inosine (Table 2). These results are almost identical with those of native IMP dehydrogenase from B. cereus.\(^9\) Therefore, IMPDH of B. cereus is overproduced by the pET system.

The impdh gene of B. cereus was cloned into E. coli for the first time, and some properties of the overproduced enzyme were investigated. It has been reported that Salmonella typhimurium Put A protein, pyrroline-5-carboxylate dehydrogenase, regulates gene expression by binding specifically to multiple sites in the put control region in vitro.\(^{27,28}\) Several workers have reported that impdh gene expression is regulated by the intracellular level of guanine nucleotides.\(^{27,28}\) B. cereus IMPDH was isolated as a DNA-binding protein in vitro at the sensitive stage for sporulation, suggesting the novel function of IMPDH as a transcription regulator in B. cereus.\(^{12}\) We are trying to identify the specific DNA binding site of B. cereus native and overproduced IMPDHs and to clarify the function of the enzyme as a DNA-binding protein.

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