Short Communication

Characterization of a thermostable adenosine 5'-monophosphate deaminase gene in *Streptomyces murinus*

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Adenosine 5'-monophosphate (AMP) deaminase (AMPD; EC 3.5.4.6), an enzyme that catalyzes the deamination of AMP to produce inosine 5'-monophosphate (IMP), plays an important role in purine/urate metabolism in higher eukaryotes (Morisaki and Morisaki, 2008). In humans, metabolic myopathy is known to be associated with a lack of AMPD in skeletal muscle (Morisaki et al., 1992; Rubio et al., 2000). In plants, AMPD has been shown to be a target of herbicides (Dancer et al., 1997; Sabina et al., 2007). In addition to the important physiological and metabolic functions of AMPD in living cells, AMPD from *Aspergillus melleus* has been applied to the production of yeast extract with enhanced taste (Tabata et al., 1994). It converts tasteless AMP, which is produced by the digestion of yeast RNA by RNases, into IMP, which has an "umami" flavor. Thus, AMPD is an industrially important enzyme. Amano Enzyme, Inc. carried out microbial screening for thermostable AMPDs that would be more suitable for industrial application. As a result, two thermostable AMPDs

were found, one from *Aspergillus fumigatus* (Moriguchi, 2006) and one from *Streptomyces murinus* IFO 14082 (= NBRC 14082) (Mizuguchi et al., 2005). This study deals with the thermostable AMPD gene found from *S. murinus*. To the best of our knowledge, this is the first bacterial AMPD to be identified.

S. murinus is known to produce a thermostable xylose isomerase, which has been used for isomerization in sugar manufacturing for many years (Ashby et al., 1987; Bandlish et al., 2002). As mentioned above, Mizuguchi et al. (2005) found a thermostable AMPD that is not denatured and that remains active at up to 65°C at its optimum pH of 5.6 in the culture medium of *S. murinus*. The AMPD appeared to function as a monomer and the molecular weight of the protein was estimated to be 48±2 kDa and 60±3 kDa by gel filtration and SDS-PAGE, respectively (Mizuguchi et al., 2005). The N-terminal and internal amino acid sequences of the purified AMPD were determined by protein sequencing (Mizuguchi et al., 2005). The 3.8 kb *NotI* DNA fragment, which was assumed to contain the whole AMPD gene, was cloned by conventional methods, including PCR amplification of part of the AMPD gene, Southern hybridization, and colony hybridization (Mizuguchi et al., 2005). When the resulting plasmid was introduced into a conventional *Streptomyces* host, *Streptomyces lividans* TK24, the AMPD protein was

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produced extracellularly (2,912 units per 1 μ l culture supernatant), indicating that the cloned DNA fragment included the complete AMPD gene (Mizuguchi et al., 2005). This result suggests that the AMPD protein is secreted by the original *S. murinus* strain as well as the recombinant *S. lividans* strain. The nucleotide sequence of the 3.8 kb DNA fragment was determined and the AMPD gene was identified (Mizuguchi et al., 2005, accession number DD250132). However, a detailed analysis of the translational and transcriptional start points of the AMPD gene remained to be performed, and in this study, we report our efforts to determine these transcriptional and translational start points. This work will aid the future development of large-scale production of the thermostable AMPD in its original host and/or in heterologous strains.

We analyzed the 3.8-kb DNA fragment using the Frame Plot program (Ishikawa and Hotta, 1999) (Fig. 1). In the upstream region of the AMPD gene, two open reading frames (*orf1* and *orf2*) exist in the opposite direction to this gene. ORF1 (305 amino acids) shows a high amino acid sequence similarity (40–70% identity) with putative hydrolases from actinobacteria. ORF2 (83 amino acids) also shows a high amino acid sequence similarity (40–70% identity) with conserved hypothetical proteins from several *Streptomyces* species. In the downstream region of the AMPD gene, a truncated open reading frame (*orf3*) exists in the same direction as the AMPD gene. The N-terminal 95 amino acid portion of ORF3 shows a high amino acid sequence similarity (65–75% identity) with N-terminal portions of several conserved hypothetical proteins from *Streptomyces* species. Because there is a 325 bp non-coding sequence between the stop codon of the AMPD

gene and the probable start codon of *orf3*, *orf3* is assumed to have its own promoter. An inverted repeat sequence (5'-TTCCGGCCGCGGCCGTGCGGACGGC CGCGGCCGGA-3'), which may function as the transcriptional terminator of the AMPD gene, is located just 5 nucleotides downstream of the stop codon of the AMPD gene. Thus, the AMPD gene appears to be transcribed monocistronically.

For transcriptional analysis, *S. murinus* was cultivated in 75 ml AMPD-producing medium (2.0% defatted soy flour, 0.5% yeast extract, 0.3% NaCl, 0.1% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 3.0% soluble starch, pH 5.7) in a 300 ml Erlenmeyer flask at 30°C for 36 h. The total RNA was then isolated with an RNAqueous kit (Ambion) and S1 nuclease mapping was performed using methods described previously (Bibb et al., 1985; Kelemen et al., 1998; Tezuka et al., 2009). Hybridization probes were prepared by PCR with a pair of ^{32}P -labelled (S1-R; 5'-GGACAGCAGGGACAGGACAC-3', see Fig. 2A and C) and non-labeled (S1-F; 5'-TCCCGCGC GCACCGCCCTTCCG-3', see Fig. 2C) primers. Only one band (approximately 80 nucleotides) was detected as a protected DNA fragment from S1 nuclease digestion by hybridization between the probe DNA and mRNA (Fig. 2B). This indicates that the AMPD gene has only one transcriptional start point, and that it is located approximately 80 nucleotides upstream of the nucleotide that corresponds with the 5' nucleotide of the labeled primer. We then attempted to determine the exact transcriptional start point by 5'-RACE using a Full RACE Core set (TaKaRa Biochemicals), according to the manufacturer's instructions. The primers used in the 5'-RACE experiment were D-RT (5'-CTTGTCAGC TTGCC-3'), which was 5'-phosphorylated by T4 DNA

Fig. 1. Gene organization of the previously cloned 3.8 kb DNA fragment.

Arrows indicate the length and direction of open reading frames (ORFs). Three complete and one truncated ORFs were predicted by the Frame Plot program. Open and closed arrows indicate truncated and complete ORFs, respectively.

(A) Nucleotide sequence of the 5' region of the AMPD gene. The amino acid sequence is also shown below the nucleotide sequence of the coding region. The N-terminal amino acid sequence of the purified AMPD, which was previously determined by protein sequencing (Mizuguchi et al., 2005), is underlined. The position of the oligonucleotide used as a reverse (labeled) primer in S1 nuclease mapping is indicated by a horizontal arrow below the nucleotide sequence. Two possible translational start codons are indicated in boldface letters. The established transcriptional start point and the putative -10 hexamer are also indicated. (B) Low-resolution S1 nuclease mapping. RNA was prepared from *S. murinus* grown in AMPD-producing medium. M, Marker 10 (Nippongene); A, *S. murinus* RNA+Probe. (C) Schematic representation of the positions of primers used in the transcription analysis. Primers S1-F and S1-R were used for preparation of the probe DNA for S1 nuclease mapping. Primers D-R2, D-R1, D-F1, D-F2, and D-RT were used for 5'-RACE. First strand cDNA was synthesized by reverse transcription using primer D-RT. T4 DNA ligase treatment resulted in the formation of concatemers and/or circular structure of the cDNA. Using this concatemeric and/or circular cDNA as a template, a DNA fragment containing the junction was amplified by nested PCR with primers D-R1 and D-F1 (first round) and D-R2 and D-F2 (second round). The nucleotide sequence of the amplified DNA fragment was determined.

AGTTC-3') and D-R2 (5'-TTGGGGAGCTGCCGAAG AAG-3'), both of which were used for second PCR (see Fig. 2C for the position of each primer). The PCR products were cloned into pBluescript and the nucle-

otide sequences of five independent clones were determined. All five clones had the same sequence, and indicated that the transcriptional start point of the AMPD gene was the G located 83 nucleotides upstream of the nucleotide that corresponds with the 5' nucleotide of the labeled primer (Fig. 2A). Hereafter, this G is referred to as +1. A -10 hexamer (GATCAT) that somewhat resembles the conserved housekeeping -10 hexamer (TAGPuPuT, where Pu is A or G, recognized by the σ^{HrdB} -containing RNA polymerase holoenzyme (Brown et al. 1992; Shiina et al. 1991; Strohl, 1992) was found in the appropriate position (Fig. 2A). However, no conserved housekeeping -35 hexamer sequence (TTGACPu) was found.

Before analysis of the translational start points, we constructed an improved system for the heterologous expression of the AMPD gene. The AMPD gene, with its upstream region of approximately 600 bp and downstream region of 62 bp, was amplified by PCR using the primers AMPD1 (5'-TGGGAGCTCACTGGGCTGTATACCG-3') and AMPD2 (5'-TGGGAGCTCTACTTCTCGCCAATCGATGCATTCCG-3'), both of which contained a *SacI* site (indicated by underlining). The amplified fragment was cloned into pBluescriptII SK and sequenced to confirm the absence of errors introduced during PCR. The *SacI* fragment was excised from this plasmid and ligated with *SacI*-digested pIJ702 (a high copy-number plasmid) (Katz et al., 1983). The ligated plasmid was introduced into *S. lividans* TK21 by protoplast transformation. Plasmids were extracted from several transformants and the insertion of the *SacI* fragment was confirmed by PCR amplification using appropriate primers. A transformant containing the correctly constructed plasmid (pIJ702-AMPD), on which the AMPD gene was located in the opposite direction from the *melC* promoter that was located near the *SacI* site, was selected. The AMPD production was examined as follows. The transformant was cultivated in 75 ml AMPD-producing medium containing 50 $\mu\text{g/ml}$ thiostrepton in a 300 ml Erlenmeyer flask at 30°C for 5 days with rotary shaking (at 150 rpm). The AMPD activity of the culture supernatant was measured spectroscopically by monitoring the decrease in the amount of AMP in the reaction at 265 nm. The reaction mixture consisted of 4.25 mM AMP-2Na (Sigma-Aldrich), 33.5 mM potassium phosphate buffer (pH 5.6) and the culture supernatant (0.5 ml) in a total volume of 2 ml. The reaction mixture was incubated for 15 min at 37°C, and 2 ml of 2% (v/v) perchloric acid solution was added to

quench the enzymatic reaction. The quantity of AMP consumed during the reaction was then determined. The AMPD required to catalyze the consumption of AMP at a rate of a loss of 0.001 AU (absorbance unit) at 265 nm in 1 h was defined as one unit. The transformant showed a higher AMPD activity ($37,100 \pm 490$ units per 1 μl culture supernatant, $n=2$) than both the previously described recombinant *S. lividans* TK24 strain (2,912 units) (Mizuguchi et al., 2005) and the wild-type *S. murinus* ($1,970 \pm 160$ units, $n=3$). No AMPD activity was detected from *S. lividans* TK21 harboring the empty vector pIJ702. In the previous study (Mizuguchi et al., 2005), an *E. coli-Streptomyces* shuttle plasmid, which was constructed from pUC19 and pIJ702, was used for the expression of the AMPD gene in *S. lividans*. In contrast, in this study, the 2.3-kb DNA fragment including the AMPD gene was directly cloned into pIJ702. Because the copy number of a shuttle plasmid in *Streptomyces* is generally much less than that of the original plasmid, it was highly expectable that the expression plasmid constructed in this study should be superior to the previous one in the amount of AMPD produced.

By using this heterologous expression system, the translational start point of the AMPD gene was analyzed. The transcriptional start point, determined as described above, suggested two possible start codons: $^1\text{GTG}^3$ (+1 to +3) and $^{49}\text{GTG}^{51}$ (+49 to +51) (Fig. 2A). To examine which of these is the functional start codon, a point mutation ($\text{GTG} \rightarrow \text{GTC}$) was introduced onto each of the two putative start codons of the AMPD gene on plasmid pIJ702-AMPD, using the QuikChange multisite-directed mutagenesis kit (Stratagene), according to the supplier's instructions. The following primers were used: G1650C_S (5'-GTCGTTCCGGCCGTcCTCGGCACCCTCAG-3'), G1650C_A (5'-CTGAGGGTGCCGAGgACGGCCGGAACGAC-3'), G1602C_S (5'-CTGATCATGCACCTGTcAGTCTGCACACCC-3'), and G1602C_A (5'-GGGTGTGCAGACTgACAGGTGCATGATCAG-3'), with altered nucleotides shown in lower case. Plasmid pIJ702-AMPD was used as the template. The introduction of mutations and the absence of errors introduced during PCR in the coding sequences were confirmed by nucleotide sequencing. The *S. lividans* strain harboring pIJ702-AMPD with a mutation in the upstream putative start codon ($^1\text{GTG}^3 \rightarrow ^1\text{GTC}^3$) was not able to produce the AMPD protein, as determined by SDS-PAGE (Fig. 3). In contrast, the *S. lividans* strain harboring pIJ702-AMPD with a mutation

Fig. 3. Production of AMPD from recombinant *S. lividans* strains, as determined by SDS-PAGE.

A time course (4, 5 and 6 days after inoculation) of the extracellular production of AMPD from recombinant *S. lividans* strains was generated; each strain contains pIJ702-AMPD with the mutation indicated. In the lanes indicated by M, a molecular mass marker (GE Healthcare) was applied. The samples were prepared by mixing 15 μ l culture supernatant with 5 μ l 4 $\times$ SDS sample buffer (24% 2-mercaptoethanol, 8.0% SDS, 40.0% glycerol, 0.1% bromophenol blue and 0.2 M Tris-HCl, pH 6.8). The sample mixtures (20 μ l) were boiled for 3 min and applied to SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. Recombinant AMPD proteins are indicated by an arrow.

in the downstream putative start codon ($^{49}\text{GTG}^{51} \rightarrow ^{49}\text{GTC}^{51}$) efficiently produced AMPD, similarly to the *S. lividans* strain harboring pIJ702-AMPD with no mutation (Fig. 3). These observations show that the upstream $^1\text{GTG}^3$ functions as the start codon of the AMPD gene in the heterologous host *S. lividans*. The functional start codon should be the upstream $^1\text{GTG}^3$ also in *S. murinus*, because the transcription and translation systems are essentially common among *Streptomyces* species. Thus, the translational start point of the AMPD gene was shown to be same as the transcriptional start point, indicating that the AMPD gene is transcribed as a leaderless mRNA. In *Streptomyces*, several genes have been reported to be transcribed as leaderless mRNAs (Bibb et al., 1985; Horinouchi et al., 1987; Jones et al., 1992; Wu and Janssen, 1997).

The determination of the translational start point allowed us to examine the possible secretion signal peptide of the AMPD. The SignalP program (<http://www.cbs.dtu.dk/services/SignalP/>) predicted that AMPD has a signal peptide (probability score: 1). Although

the most likely cleavage site was predicted to be between Ala-39 and Ala-40 (probability score: 0.528), we speculated that the actual cleavage site may be between Ala-44 and Ala-45, as the N-terminal end of the native enzyme purified from the *S. murinus* culture broth has been found to be Ala-45. It is also possible that a protease(s) removes a short peptide from the pro-AMPD to generate the mature AMPD. The secretion of AMPD appears to be very efficient, and a large quantity of AMPD was secreted from the recombinant *S. lividans* strain. AMPD represented more than 95% of the extracellular protein secreted by this strain (Fig. 3). The extracellular production of AMPD is advantageous to its industrial production, with the preparation of the enzyme from culture medium being relatively easy, allowing production costs to be reduced.

We searched for the AMPD homologs in the NCBI database with the BLAST-P program. Several *Streptomyces* species, including *S. griseus* XylebKG-1, *S. roseosporus* NRRL 11379, *Streptomyces* sp. SirexAA-E, and *S. flavogriseus* ATCC 33331, have putative AMPDs, which show entire amino acid sequence similarity (66–69% identity) to the *S. murinus* AMPD. These putative *Streptomyces* AMPDs have a probable N-terminal signal sequence and seem to be secreted into the medium. In addition to these putative *Streptomyces* AMPDs, more than 50 AMPD homologs that show entire amino acid sequence similarity (>50% identity) were found not only in gram positive bacteria but also in gram negative bacteria including *Caulobacter*, *Acetobacter*, *Gluconacetobacter*, *Zymomonas*, *Azospirillum*, *Xanthomonas*, *Legionella* and several other genera. The SignalP program predicted that some of these AMPD homologs have a putative N-terminal signal sequence but some of them do not. The AMPD activity of these homologs remains to be examined.

In conclusion, we have revealed that the *S. murinus* AMPD is synthesized as a polypeptide of 535 amino acid residues and that it has a signal peptide (which is likely to be composed of 44 amino acids). The transcriptional start point of the AMPD gene is the same as the translational start point; the gene is therefore transcribed as a leaderless mRNA. These findings will be important for the future development of large-scale production of this thermostable, industrially useful AMPD, either in its original host and/or in heterologous strains.

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