Cloning and Expression of the N,N-Dimethylformamidase Gene from *Alcaligenes* sp. Strain KUFA-1

Yoshie HASEGAWA,† Tai TOKUYAMA, and Hiroaki IWAKI

Department of Biotechnology, Faculty of Engineering and High Technology Research Center, Kansai University, Suita, Osaka 564-8680, Japan

Received June 14, 1999; Accepted August 30, 1999

_N,N-Dimethylformamidase (DMFase) from *Alcaligenes* sp. strain KUFA-1, a bacterium that can grow on N,N-dimethylformamide (DMF) as the sole carbon and nitrogen source, catalyzes the first step of the DMF degradation. The DMFase gene *dmfA1A2* was cloned in *Escherichia coli*, and its nucleotides were sequenced. The deduced amino acid sequence of the enzyme consisted of two α- and two β-subunits with 132 and 762 amino acids, respectively, and had little similarity to sequences in protein databases, including various amidases. The protein may be a new kind of amidase. DMFase activity was detected in *E. coli* cells transformed with an expression plasmid of the cloned DMFase gene. The properties of recombinant DMFase purified from *E. coli* were identical to those of *Alcaligenes* DMFase.

Key words: *Alcaligenes* sp.; _N,N-*dimethylformamidase gene; gene cloning; nucleotide sequencing

_N,N-Dimethylformamide (DMF) is widely used in the chemical industry as, for example, a solvent for polycrystalline fibers and in the synthesis of organic compounds. Attention has been paid to the possible environmental pollution by and toxicity of DMF to human beings and other organisms_.1

SchäR et al. isolated and characterized of a DMF-hydrolyzing enzyme (EC 3.5.1.--) from *Pseudomonas* sp. strain DMF 3/3.2 This strain hydrolyzed DMF to dimethylamine and formate by _N,N*-dimethylformamidase (DMFase). These intermediates are metabolized to ammonia and carbon dioxide by known metabolic pathways of methylotrophs. Total molecular mass of _Pseudomonas_ DMFase was reported to be 250 kDa and is composed of two light-chain (each 15 kDa) and two heavy-chain (each 105 kDa) subunits. DMFase has a narrow substrate spectrum. It preferentially hydrolyses substituted short-chain aliphatic amides.

We isolated DMF-utilizing *Alcaligenes* sp. strain KUFA-1 from activated sludge and purified DMFase from cells of this strain KUFA-1.3 The enzyme has an 200 kDa molecular mass and is composed of two light-chains (16 kDa) and two heavy-chains (84 kDa). *Alcaligenes* and *Pseudomonas* DMFases have the same oligomeric form, αββ, but they are different in size and enzymatic properties.

In this paper, the cloning and sequencing of the *Alcaligenes* DMFase gene are described. The *Alcaligenes* DMFase gene was expressed in *E. coli* and the enzyme was characterized.

Materials and Methods

_Bacterial strains, plasmids, and media. _Alcaligenes_ sp. strain KUFA-1 was used for cloning of the DMFase gene. *Escherichia coli* XL1-Blue [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacF′ proAB lacY1 ZAM15 Tn10 (Tet’)] (Strategene, La Jolla, CA, USA)6 and *E. coli* JM109 [recA1 ΔlaczproAB] endA1 gyrA96 thiI hsdR17 relA1 supE44 (F′ traD36 proAB lacY1 ZAM15) were used as hosts. The plasmid pUC187 was used as the cloning vector.

*Alcaligenes* sp. strain KUFA-1 was grown at 30°C with aeration in the liquid medium described previously.3 *E. coli* strains were cultured aerobically at 30 or 37°C in Luria-Bertani (LB) broth, that contained 100 mg/ml ampicillin when appropriate.8 Plasmid pSD80, a third-generation derivative of the expression vector pKK223-3 (Pharmacia Biotechnology, Uppsala, Sweden),7 was used as the vector for expression of the cloned DMFase gene.

_Determination of N-terminal amino acids sequence. _DMFase was purified from *Alcaligenes* sp. strain KUFA-1 as described previously.9 For sequencing of the N-terminal amino acids, the protein bands of the ß- and ß-subunits of DMFase were separated by SDS-PAGE (10% polyacrylamide gel) and transferred onto a polyvinylidene difluoride membrane with a blotting apparatus (Bio-Rad, Trans-blot SD Semi-Dry Transfer Cell). The membrane was stained with 0.025% Coomasie Brilliant Blue R-250 and destained with 100% methanol. The protein bands were excised and the N-terminal amino acids were sequenced by the automated Edman degradation with a pulsed-liquid sequencer (model 476A, Applied Biosystems, Foster City, CA, USA).

Isolation of DNA and PCR for detection of the DMFase gene. Genomic DNA of _Alcaligenes_ was purified from 10-ml stationary-phase cultures as described elsewhere.8 On the basis of the N-terminal sequences of the ß- and ß-subunits of DMFase, four DNA primers (designated H-S, H-A, L-S, and L-A) were synthesized by Amersham Pharmacia Biotech. The nucleo-
tide sequences of primers H-S (corresponding to the amino acid residues MKDIAIRG) and H-A (GETIRFYYV) were based on the N-terminal sequence of the β subunit, and the sequences of L-S (ADWYAFY) and L-A (AD-WYAFY) were based on the sequence of the α-subunit. Primer H-S was 5'-ATGAAARGATIGCATIAM-GIGG-3' (sense strand), primer H-A was 5'-ACIG-TRAACDDATIGGTYCC-3' (antisense), primer L-S was 5'-GGCIGAYTTGGATYGCYITTYTA-3' (sense), and primer L-A was 5'-RTARAAIGCRTACCCATCGC-G-3' (antisense) (R: G or A; Y: T or C; M: A or C; I: K; G or T; D: A, G, or T; I: inosine, fitting with all bases). Two sets of primers (H-S and L-A, or L-S and H-A) were used for PCR. Amplification by PCR was done in a reaction mixture (100 μl) containing 1.0 μg of Alcaligenes genomic DNA as a template, two primers (0.5 μM each), four deoxyribonucleoside triphosphates (dNTPs); (200 μM each), 1.5 mM MgCl₂, 50 mM KCl, and 2.5 U Taq polymerase (Life Technologies, Inc., Rockville, MD, USA.) in 10 mM Tris-HCl (pH 8.3). DNA was denatured at 94°C for 1 min, the primer-annealing step was at 50°C for 1 min, and the primer-extension step was at 72°C for 1 min. The reaction ran for 30 cycles. The PCR product was purified on an agarose gel and labeled with the digoxigenin-11-UTP system (Boehringer Mannheim GmbH) by the manufacturer's instructions for use as a probe in Southern and colony hybridization.

Construction of gene libraries. The total DNA of Alcaligenes sp. strain KUFA-1 was digested with various restriction enzymes independently, electrophoresed on a 0.8% agarose gel, transferred onto a Hybond-N membrane (Amersham) with a capillary array, and hybridized with the probe. The signals were analyzed on X-Omat AR x-ray films (Kodak, Rochester, NY, USA.). DNA fragments with lengths from 6.5- to 7.5-kb cut by HinDIll were obtained by agarose gel electrophoresis and DNA extraction as described by He et al. The DNA fragments were ligated to the dephosphorylated HinDIll site of pUC18 and introduced into E. coli XL1-Blue. Colonies were hybridized with the probe and clones with the strong signal were selected.

Nucleotide sequencing. Nucleotide sequences of both strands of the PCR product and the cloned DMFase gene were identified by dideoxynucleotide chain termina-
tion with an automated sequencer (model 310 Genetic Analyzer, Applied Biosystems) with the cycle sequencing protocol. The nucleotide sequence to be identified in the cloned DMFase gene was synthesized as the next primer for sequencing of the next downstream portion. Sequencing was done in steps. Synthetic primers were purchased from Amersham. The DNA sequences and predicted amino acid sequences were analyzed with the GENETYX-MAC Program (Software Development, Tokyo). The deduced amino acid sequences of open reading frames were compared with databases by use of the BLAST program.

Construction of an expression plasmid of the DMFase gene. DNA fragments carrying putative genes for DMFase were amplified by Vent DNA polymerase (New England BioLabs, Inc., Beverly, MA, USA) with the HLF-HLR primer set for the amplification of αβ-subunit genes (dmfA1 and dmfA2). The sequences were 5’-TCCTCCGGCGACATGCCAACAAGGG-3’ for the HLF primer and 5’-AAAACCTGCG-CGACGCAGCGGATATCGTG-3’ for the HLR primer. The underlined sequences are Smal and PstI restriction sites constructed to facilitate cloning. The amplification conditions were 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, for 30 cycles. The PCR products were digested with Smal and PstI, and the resultant fragments were ligated into the Smal and PstI sites of pSD80. The resulting plasmid, designated pDMF101, had been prepared from transformed E. coli JM109, and insertion was confirmed by sequencing of the entire insert.

Cultivation of E. coli harboring the expression plasmid and purification of the DMFase. E. coli JM109 cells harboring pDMF101 were grown in 100 ml of LB medium containing ampicillin (100 μg/ml) at 30°C. When the culture reached an A₆₆₀ of 0.4 to 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. The cells were further cultured for 3 h. The cells were harvested, and washed twice with 50 mM phosphate buffer, pH 7.2. The cells were resuspended in a 1/20 of the original culture volume of the same buffer and disrupted sonically by four 20-s bursts (Sonifire 250, Banson, Danbury, CT, USA). Unbroken cells and cell debris were removed by centrifugation at 18,000 × g for 30 min at 4°C. The supernatant was assayed for DMFase and treated by SDS-PAGE for detection of the enzyme protein. All purification procedures were done at 4°C. A typical purification procedure for cloned DMFase was as follows.

The crude extract obtained from about 10 g (wet weight) was fractionated with (NH₄)₂SO₄, and the fractions at 60–70% saturation was collected. After dialysis of the fraction, the enzyme solution was put on a DEAE Sephadex A-50 column (2.6 × 23 cm). The column was washed with 250 ml of the buffer containing 0.1 M NaCl, and the enzyme was eluted with a linear gradient of 0.1 to 0.5 M NaCl with a total volume of 450 ml. The active fractions were dialyzed against 50 mM phosphate buffer (pH 8.0), and put on a DEAE-cellulose column (2.5 × 15 cm) equilibrated with the same buffer containing 0.1 M NaCl. The enzyme was eluted with a linear gradient of 0.1 to 0.5 M NaCl with a total volume of 450 ml. The active fractions were concentrated with use of polyethylene glycol 20,000.

Enzyme and protein assays. DMFase activity was assayed by the alkylamine-specific color reaction. The standard assay system contained 100 μl of 50 mM phosphate buffer (pH 7.0), 10 μl of DMF, and 90 μl of enzyme solution. The reaction was in 1.5-ml Eppendorf tubes for 30 min at 37°C and was stopped by the addition of 50 μl of 15% (w/v) trichloroacetic acid. One unit of enzyme activity is defined as the amount of enzyme
N,N-Dimethylformamidase Gene in *Actinomycetes* sp.

```
CTGCAGCCAGCCGGACATCGCAATACTCAGAACCGAACCGAGCTACGCTGAACGCTATC
CCGGCTTCTGTCACCCCTAATCCTGCAGAAGCCGAGCCGAGGAGATCTGGCTCAGCT
-35
CTGAGGACGTGCTAGTTACGTGCTAGCTGTACGGAGCTGCTAGCTGTACGGAGCTGCT
-10
SB

ACTTATTGTAACACACCCGGACATCGGACGGGACAGGACGCCGAACCTTGCGCTACAGGAGG
IDEPHELPEIVEHANFPFGYRKHPSPYLPQRV

CACATCTATATGTCATGCACCCGGACCTCTGGTCAGGAAGATCCGTATTCACGTATCCGGT
IHYFRMQLQPTFYPRTMSREREVWDAYRATITR

GAATCTGGCGAGCTGGCGAGTTGCGGAGTGAACAGAGCTGGCGAGCTGGCGAGAT
EFGELEMPLEGDERFKEETEEAEAHMVFLRRIED

GTGCGCCAGCTGCTGATGCCCTATGCTTTCACGCAGATTCTACAGAGGGGGAGAACATC
VRAELA*

SD

GCCCAACGGCTGCAACTGAGAACAGGACTCCCTCTTACGCGACGCACCAGAGAACAGGACCT
RPSVATGETIFVYVSAENTRGTDFDAELVRL

TCAATGCGGCAATTGGGCTGGGCTGGGATTACAGAGGAGAGGACTTCTAGCTGAGGAGGCT
THGDNSNAEAGKSDLEGQYPARFQ

GACCAAGTGGTCAATGGTGCGCTCGTGGCTGAGGGATACGCTCCGCGTGGCTGGCTG
RTQFGSYVVEVADBPDAGLQPDGAFSVALFLVLW

CGACACGGCGGGTTCGCTGAGGAGCTGCTGACGCGCTGAGGGATACGCTCCGCGTGGCTG
STTPSRGRQGIAASRWNDEQSGWSNLAIEDG

GGCTGCTGTTTACGAGCTGAGGAGCTGCTGACGCGCTGAGGGATACGCTCCGCGTGGCTG
RUVFTIGDGSVRSSVRDSRPLFQOQWSYI

CGGTGTTTACGAGCTGAGGAGCTGCTGACGCGCTGAGGGATACGCTCCGCGTGGCTG
TGVYDPEKKEQLRYQKYKSVVNRTNSRFGLVV

GGCTGAGTTATCGAGATTTGCTGAGGAGCTGCTGACGCGCTGAGGGATACGCTCCGCGTGGCTG
PLDSDCAVSADATVKAADSETSLLILLAGE

CAAGCGCCGACGGACGGCCGAGTACGAGACGCGTCGGCAGTTAAGAGGCCGCAGTGCGGC
AAQQDGRTRWCIAHNYNGKVDAPRIYGCGALQ

ACCATGCGGCAATTGGGCTGGGCTGGGATTACAGAGGAGAGGACTTCTAGCTGAGGAGGCT
DDEAELSRAEPISLRAHWDFASSAGIGLNN

GGATCCCAACGGCTATCGCTGATCGCTGATCGCGCTGAGGGATACGCTCCGCGTGGCTG
GIPTDHVVDGASNGNHGRCNGMNPDRSTGW

ACTGCGGAGCTAGGGAGAACCTTTACACTTCTACGCGCCGAGAAGCTAGCTCGCTG
NWGDGHEENHPQEQYAGALWDPHDCLDDCR

GGGGAATTTGCGGCTTACCGGCTGAGGGGCCTGAAAAGAGCTTTACACGCGCGGTAGACGAG
WEKDFEFVERTPSDFYAVKIRYEDETY

TTGGCAGCTGGTCTTTCGCCCGCCGCAAGCCACAGCCGCCGCGATCCGTCGACAGCAGC
IFFFVLPRRGTAATAAPTILVIASLSTLSYLAN

AGCAGATATCGCAACAGGGCAGGATACGCGGGCTGGCCTACTACGCGCTGCGCTGAGG
EQIMHKAIDIQVAGVHTLPVLEDVHELKNN

TGAGCTATTACGCGCTGCACTTCTATATGCTGACGCGCTGAGGGATACGCTCCGCGG
LSTYGLSLYIDGMDRGVQYVTSTWRPPIMNL

GCCCGGCAACCGTATCTGAGTTTCTGCGCTCTGCTGAGGCTCCCGCCGCTAGCTACCTGAGTG
RPFKQRQGFGSIWELPADLDHLHDWNLNNHNPFE

AGCAGATATCGCAACAGGGCAGGATACGCGGGCTGGCCTACTACGCGCTGCGCTGAGG
YDVAETEHLNDQGAEELRKYKVLTGSHPE
```

(continued)
catalyzing the hydrolysis of 1 μmol of DMF/min under the assay conditions. Protein concentration was assayed by the Bradford method(3) with bovine serum albumin as the standard.

**Nucleotide sequence accession number.** The nucleotide sequence reported here was submitted to the DDBJ/GenBank/EMBL DNA databases and given accession number AB028874.

**Results and Discussion**

**N-Terminal sequence of DMFase**

The sequence of the α-subunit polypeptide was [TEASESCVRDPSNYDRSADWYAFY...] and that of the β-subunit polypeptide was [MKDIAIR-GYCGRPSVATGETIRFVY...]. The Flavobacterium glycosylasparaginase, an amidasie, is a heterodimer with an estimated molecular mass of 38 kDa; it consists of an α-subunit (18 kDa) and β-subunit (16 kDa).(14) In all known (glycosyl)asparaginases, the amino-terminal threonine residue of β-subunit is at an active site residue. The α-subunit of Alcaligenes DMFase had threonine at the amino-terminus residue.

**Cloning and sequencing of the DMFase gene**

Based on the N-terminal sequence of α- and β-subunit of DMFase, the oligonucleotide primers for PCR amplification were designed as described in Materials and Methods. A 400-bp-long product was obtained by PCR only when L-S and H-A were the primers in the amplification. The amino acid sequence, MKDIAIR-GYCGRPSVAT, deduced from the DNA sequence of this PCR product was identical to the N-terminal sequence of the β-subunit of DMFase as described above.

The 400-bp-long PCR product was used as a probe for the Southern analysis of the Alcaligenes DMFase gene. On the basis of results of Southern analysis, 6.5 to 7.5 kb long HindIII fragments of Alcaligenes total DNA were ligated to the HindIII site of pUC18 and used to transform E. coli XL1-Blue cells. One of the 250 colonies harboring the recombinant plasmid demonstrated positive hybridization reaction with the probe DNA. The plasmid from this clone, which had an Alcaligenes HindIII fragment 7.0 kb long inserted in pUC18. This plasmid was designated pDMF100. Nucleotide sequence of the 7.0-kb HindIII fragment were determined and the deduced amino acid sequences for DMFase gene (Fig. 1). We found that the α-subunit polypeptide was encoded at a region 38 bp upstream of the region encoding the β-subunit polypeptide. We designated the orfs for the α- and β-subunits of DMFase are *dmfA1* and *dmfA2*, respectively.

The *dmfA1* gene for the α-subunit started with an ATG codon at nucleotide +1 and ended with TGA at position 397. These 396 nucleotides encoded a polypeptide of 132 amino acids with a deduced molecular mass of 16,031 Da. The *dmfA2* gene for the β-subunit started with an ATG at 435 and ended with TGA at 2721. This 2286-bp sequence encodes a polypeptide of 762 amino acids with a deduced molecular mass of 84,699 Da. Both orfs are preceded by consensus sequence for ribosome-binding, GGAGGAGA in *dmfA1* and GGAGAAG in *dmfA2*. Both *dmfA1* and *dmfA2* genes were typical of *Alcaligenes* genes in their G+C contents (58.9% and 59.5%, respectively). Sequences resembling the *E. coli* −35 (TTGACA) and −10 (TATAAT) promoter

---

![Fig. 1. Nucleotide Sequence of DMFase Gene of *Alcaligenes* sp. Strain FUKA-1 and Its Deduced Amino Acid Sequence.](image-url)
regions were found at nucleotide positions from −47 to −42 and from −26 to −21, respectively. Inverted repeat sequences were found at positions 2735–2744 and 2752–2761 in the termination region. This sequence might be involved in the termination of translation.

A search for sequences similar to the deduced amino acid sequences of dmfA1 and dmfA2 was done in the Swiss protein database. The deduced sequences had little similarity to any other hydrolase, suggesting that the enzyme is a new kind of amidase.

Expression of the D MFase gene in E. coli

A plasmid, pDMF101, carrying the complete dmfA1- dmfA2 sequence was constructed as described in Materials and Methods. The E. coli JM109 clone harboring pDMF101 had shown DMFase activity, whereas no DMFase activity was detected in E. coli JM109 cells without harboring plasmids or with vector plasmid (pSD80) alone. Sonicated extracts of E. coli cells harboring pDMF101 were examined on the SDS-PAGE (Fig. 2A). Both α- and β-subunits were detected in E. coli cells harboring pDMF101 when cultured in the presence of IPTG (lane 1), but corresponding bands were not found in the similar extracts of E. coli JM109 cells harboring pSD80 cultured in the presence of IPTG (lane 2). In addition, E. coli JM109 clones harboring a dmfA1- or dmfA2-expression plasmid constructed with pSD80 did not have DMFase activity. These results indicated that both the dmfA1 and dmfA2 genes were needed for DMFase activity. However, no DMFase activity was detected in the mixed cell extracts from E. coli JM109 cells harboring the dmfA1- and dmfA2-expression plasmid.

Purification and characterization of DMFase from the recombinant E. coli cells

DMFase was purified from a cell extract of E. coli JM109 harboring pDMF101. Typical results of purification procedures for a DMFase are summarized in Table 1. The DMFase was purified 37.2-fold compared with the cell extract and recovered in 15.0% yield. The purified enzyme gave two bands on SDS-PAGE and their apparent molecular mass was 16 kDa of α-subunits and 84 kDa of β-subunit, respectively (Fig. 2B). These values corresponded to the molecular mass calculated from the nucleotide sequence. By using a Superose 12 gel filtration, the apparent molecular mass of the purified enzyme was 200,000, indicating the enzyme is an αβ2-type enzyme (Fig. 3).

The purified DMFase was stable against being heated at 50°C for 30 min. Exposure to temperatures of 60°C or more for 30 min rapidly inactivated the enzyme. The activity was maximum at 50–55°C. The enzyme activity at 55°C was 5 times that at 25°C. The optimum pH for the activity was from 6.0 to 7.0. The purified enzyme interacted specifically with DMF as substrate. Besides the natural substrate DMF (relative activity of 100%; 30.85 U/mg), only N,N-dimethylacetamide (38.5%) and N,N-diethylacetamide (40.1%) were reasonably good substrates of the 12 substrates we tried. The enzymatic properties of the cloned DMFase were
Table 1. Purification of DMFase from *Escherichia coli* Harboring pDMF101

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>248</td>
<td>206</td>
<td>0.83</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>60-70% (NH$_4$)$_2$SO$_4$</td>
<td>105</td>
<td>164</td>
<td>1.56</td>
<td>79.6</td>
<td>1.9</td>
</tr>
<tr>
<td>DEAE Sephadex A-50</td>
<td>9.9</td>
<td>63.8</td>
<td>6.44</td>
<td>31.0</td>
<td>7.8</td>
</tr>
<tr>
<td>DEAE Cellulose DE52</td>
<td>1.0</td>
<td>30.9</td>
<td>30.9</td>
<td>15.0</td>
<td>37.2</td>
</tr>
</tbody>
</table>

![Fig. 3. Estimation by Gel Filtration of Molecular Mass of DMFase from *E. coli* Harboring pDMF101.](image)

Calibration of a Superose 12 gel filtration column was done with the native proteins listed. $K_a$ values for each protein were calculated from equation $K_a = (V_e - V_b)/(V_i - V_b)$. $V_e$, elution volume for the protein, $V_b$, elution volume for Blue Dextran 2000 (6.8 ml), $V_i$, total bed volume (25.0 ml). $V_e$ for DMFase was 10.1 ml.

identical to those of DMFase from *Alcaligenes* sp. strain KUFA-1. The functions of the $\alpha$- and $\beta$-subunits are not known.

Acknowledgments

Financial support for this study was provided by a Kansai University Grant-in-Aid for Joint Research, 1999, and by the Special Research Promotion Fund administered by the Japan Foundation for the Promotion of Private School.

References


