

# Molecular Cloning, Sequencing, and Expression of the Gene Encoding Alkaline Ceramidase from *Pseudomonas aeruginosa*

CLONING OF A CERAMIDASE HOMOLOGUE FROM *MYCOBACTERIUM TUBERCULOSIS*\*

(Received for publication, June 16, 1999, and in revised form, September 18, 1999)

Nozomu Okino, Sachiyo Ichinose‡, Akira Omori‡, Shuhei Imayama§, Takashi Nakamura, and Makoto Ito¶

From the Department of Bioscience and Biotechnology, Division of Bioresource and Bioenvironmental Sciences and the §Department of Dermatology, Faculty of Medicine, Graduate School Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan and the ‡Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida 194-8511, Tokyo, Japan

We previously reported the purification and characterization of a novel type of alkaline ceramidase from *Pseudomonas aeruginosa* strain AN17 (Okino, N., Tani, M., Imayama, S., and Ito, M. (1998) *J. Biol. Chem.* 273, 14368–14373). Here, we report the molecular cloning, sequencing, and expression of the gene encoding the ceramidase of this strain. Specific oligonucleotide primers were synthesized using the peptide sequences of the purified ceramidase obtained by digestion with lysylendopeptidase and used for polymerase chain reaction. DNA fragments thus amplified were used as probes to clone the gene encoding the ceramidase from a genomic library of strain AN17. The open reading frame of 2,010 nucleotides encoded a polypeptide of 670 amino acids including a signal sequence of 24 residues, 64 residues of which matched the amino acid sequence determined for the purified enzyme. The molecular weight of the mature enzyme was estimated to be 70,767 from the deduced amino acid sequence. Expression of the ceramidase gene in *Escherichia coli*, resulted in production of a soluble enzyme with the identical N-terminal amino acid sequence. Recombinant ceramidase was purified to homogeneity from the lysate of *E. coli* cells and confirmed to be identical to the *Pseudomonas* enzyme in its specificity and other enzymatic properties. No significant sequence similarities were found in other known functional proteins including human acid ceramidase. However, we found a sequence homologous to the ceramidase in hypothetical proteins encoded in *Mycobacterium tuberculosis*, *Dictyostelium discoideum*, and *Arabidopsis thaliana*. The homologue of the ceramidase gene was thus cloned from an *M. tuberculosis* cosmid and expressed in *E. coli*, and the gene was demonstrated to encode an alkaline ceramidase. This is the first report for the cloning of an alkaline ceramidase.

Ceramidase (CDase, EC3.5.1.23)<sup>1</sup> is an enzyme that catalyzes the hydrolysis of the *N*-acyl linkage of ceramide to generate free fatty acid and sphingosine base. Since the first description of CDase activity in the rat brain by Gatt (1, 2), CDase has been found ubiquitously in mammalian tissues (3). Isoforms of CDase have been reported which mainly differ in their catalytic pH optima. Acid CDase in lysosomes plays a crucial role in the catabolism of ceramide, and an inherited deficiency of this enzyme leads to the accumulation of ceramide in lysosomes that is known as Farber disease (4). Acid CDase was purified from human urine (5), and the cDNAs encoding the enzyme have been cloned from human fibroblasts (6) and mouse brain (7). Genes encoding isoforms of CDase showing neutral to alkaline pH optima have not yet been cloned although these enzymes could function in sphingosine-mediated signal transduction pathways in eukaryotic cells (8–10).

Sphingosine, a catalytic product of ceramide generated by CDase, has recently emerged as a novel lipid biomodulator. Several lines of evidence indicated that sphingosine could modulate cell activities *via* inhibition of protein kinase C (11), modulation of Na<sup>+</sup>, K<sup>+</sup>-ATPase (12), and some protein kinases (13). Furthermore, sphingosine-1-phosphate, a phosphorylated derivative of sphingosine generated by the action of sphingosine kinase (14), was found to be involved in diverse biological processes including cell growth, survival, and motility (15–17). Recently, a new class of G protein-coupled receptors named the EDG family was cloned, some of which were identified as putative receptors for sphingosine-1-phosphate (18, 19). CDase is considered to be a rate-limiting enzyme in generation of sphingosine and might be that of sphingosine-1-phosphate because sphingosine is only produced from ceramide by the action of ceramidase and not by *de novo* synthesis (20). CDase could therefore have a critical role in signal transduction mediated by sphingosine and might be sphingosine-1-phosphate. It is interesting to note that neutral and alkaline CDases were up-regulated by growth factors (8) and cytokines (10).

In contrast to those of mammalian origin, there have been few reports on the occurrence of ceramidase in invertebrates. Recently, we purified and characterized a CDase from the Gram-negative bacterium *Pseudomonas aeruginosa*, strain AN17, isolated from a patient with atopic dermatitis (21). This CDase, the first reported from a prokaryote, showed the highest activity around pH 8.5 and required calcium ions for its

\* This work was supported in part by a Grant-in Aid for Scientific Research (B) (09460051) from the Ministry of Education, Science and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB028646.

¶ To whom correspondence should be addressed: Laboratory of Marine Biological Chemistry, Dept. of Bioscience and Biotechnology, Division of Bioresource and Bioenvironmental Sciences, Graduate School Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan. Tel.: 81-92-642-2900; Fax: 81-92-642-2900 or 81-92-642-2907; E-mail: makotoi@agr.kyushu-u.ac.jp.

<sup>1</sup> The abbreviations used are: CDase, ceramidase; IPTG, isopropylthio- $\beta$ -D-galactopyranoside; NBD, nitrobenzo-2-oxa-1,3-diazole; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; PCR, polymerase chain reaction; kbp, kilobase pair.

TABLE I

Peptide sequences of alkaline ceramidase from *P. aeruginosa* AN17

Peptide sequences were determined by Edman sequencing. Unidentified amino acids are marked X.

N-terminal	
C-118	DDLPLYRFG(L)GKADITGEAAAVGMM
Lysylendopeptidase digest	
C-86	VTXAFVTGHPK
C-89	SFGDVLQQPRESYRIG(D)K
S-90	DDLPLYRFG(L)GK
C-91	ISEIGGSTRSFEVLGT
C-121	XFLEVVNIGK

activity and was therefore identified as a novel type of alkaline CDase (21).

Here, we report the molecular cloning, sequencing, and expression of the gene encoding the alkaline CDase from *P. aeruginosa*. We also describe the cloning and expression of a CDase homologue in *Mycobacterium tuberculosis* which is known to cause tuberculosis.

#### EXPERIMENTAL PROCEDURES

**Materials**—pTV118N, pTV119N, and DNA modifying enzymes were purchased from Takara Shuzo Co., Japan. Restriction endonucleases and Ligation Pack were purchased from Nippon Gene Co., Japan. [ $\alpha$ - $^{32}$ P]dCTP was obtained from Amersham Pharmacia Biotech. D-Sphingosine and Triton X-100 were purchased from Sigma, and pre-coated Silica Gel 60 TLC plates were from Merck, Germany. *M. tuberculosis* cosmid MTC1376 was a kind gift from Dr. S. T. Cole, Institut Pasteur, France. All other reagents were of the highest purity available.

**Amino Acid Microsequencing**—CDase from *P. aeruginosa* strain AN17 was purified as described previously (21). The purified CDase was concentrated with a Y-shaped gel (160  $\times$  160  $\times$  2 mm) modified form of a funnel-shaped one (22). After the concentration, protein band localized with Coomassie Brilliant Blue was cut out, reduced with dithiothreitol, and loaded again on a well of an usual SDS-PAGE. After electrophoresis, the gel was blotted on a polyvinylidene difluoride membrane (Immobilon-P, Millipore) and stained with Coomassie Brilliant Blue. The CDase band (about 2  $\mu$ g) was cut out and treated *in situ* with lysylendopeptidase AP-1 (Wako Pure Chemical Industries, Japan). Peptides released from the membrane were fractionated with a reversed-phase high performance liquid chromatography column of C8 (RP-300, 1.0  $\times$  100 mm, Applied Biosystems) and sequenced with a pulse-liquid phase protein sequencer (Procise 492 cLc, Applied Biosystems).

**Molecular Cloning and DNA Sequencing**—General cloning techniques were carried out essentially as described by Maniatis *et al.* (23). Genomic DNA of *P. aeruginosa* strain AN17 was isolated by the method of Saito and Miura (24). Nucleotide sequences were determined by the dideoxynucleotide chain termination method with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and a DNA Sequencer (model 377A, Applied Biosystems).

**PCR Amplification**—PCR with degenerate oligonucleotides was used to amplify a DNA fragment encoding the CDase. Sense and antisense oligonucleotide primers were designed using the internal amino acid sequences of C-89 and C-91 (Table I). PCR was performed with the AN17 genomic DNA as a template in a GeneAmp PCR System 2400 (Applied Biosystems) for 40 cycles (each consisting of denaturation at 94  $^{\circ}$ C for 30 s, annealing at 51  $^{\circ}$ C for 30 s, and extension at 72  $^{\circ}$ C for 1 min) using AmpliTaq Gold (Applied Biosystems). Amplified PCR products were cloned into the pGEM T-easy vector (Promega), and their DNA sequences were determined. One PCR product (C89 s-C91a, named Probe 1) contained the sequence found in the purified CDase.

**Isolation of Genomic DNA Clones Encoding CDase**—Genomic DNA (10  $\mu$ g) was digested with various restriction endonucleases, and the digests were fractionated by 1.0% agarose gel electrophoresis by the standard method (23). DNA was transferred from agarose gels onto nylon membranes (Hybond N<sup>+</sup>, Amersham Pharmacia Biotech) according to the protocol of the manufacturer. Probe 1 was labeled with [ $\alpha$ - $^{32}$ P]dCTP using a Ready-To-Go<sup>TM</sup> DNA labeling kit (Amersham Pharmacia Biotech) and used for hybridization, which was performed in 0.5 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 7% SDS at 65  $^{\circ}$ C for 16 h. After hybridization, the membrane was washed three times with 40 mM sodium phosphate buffer, pH 7.0, containing 1% SDS at 65  $^{\circ}$ C and was exposed on an imaging plate, which was then examined after several hours using a BAS 1000 imaging analyzer (Fuji

Film, Japan). Judging from the Southern blots of the *Apa*I digest using Probe 1, only the 2.4-kbp fragment was found to contain the CDase gene. For cloning of this gene, a digest with *Apa*I was prepared using 10  $\mu$ g of genomic DNA. Restriction fragments of genomic DNA of *P. aeruginosa* strain AN17 were fractionated by preparative 1.0% agarose gel electrophoresis, and 2.4-kbp fragments were extracted from the gel. The *Apa*I fragments were ligated to the *Apa*I site of pBluescript II SK (Stratagene). The recombinant plasmids thus obtained were used to transform *Escherichia coli* DH5 $\alpha$ , which were employed for preparation of a gene library enriched with the CDase gene. Colony hybridization was performed by the standard procedure using Probe 1 (23). One clone was selected, and the plasmid in the clone was designated pSCA59.

Probe 2 was prepared by digestion of pSCA59 with *Kpn*I and *Sac*II. The probe, about 500-bp long, contained the 5'-end of the CDase gene in pSCA59 and was labeled with [ $\alpha$ - $^{32}$ P]dCTP. *Bam*HI fragments (1.9 kbp) from genomic DNA of *P. aeruginosa* strain AN17 were ligated to the *Bam*HI site of pGEM-3Z $\alpha$  (+) (Promega) and used to transform *E. coli* DH5 $\alpha$ , which were used for preparation of a gene library enriched with the 5'-end of the CDase gene. The library was screened by colony hybridization with Probe 2.

**Construction of Expression Plasmid with CDase Gene**—The vector pTV119N was treated with *Hind*III. An insert that included the 5'-end of the CDase gene was prepared by digestion of deletion mutant of clone pGCB38 (pGCB38-D13) with *Hind*III and *Bam*HI (nucleotide position, 612–1957), and purified by agarose gel electrophoresis. An insert that included the 3'-end of the CDase gene was prepared by digestion of clone pSCA59 with *Hind*III and *Bam*HI, and purified by agarose gel electrophoresis. Two inserts were ligated with vector and used to transform *E. coli* JM109. The recombinant plasmid was purified and designated pTCD11.

**Expression and Purification of Recombinant CDase**—*E. coli* JM109 cells transformed with pTCD11 were grown at 37  $^{\circ}$ C in Luria-Bertani medium containing 100  $\mu$ g/ml ampicillin until the optical density at 600 nm reached about 0.5. Then isopropylthio- $\beta$ -galactopyranoside (IPTG) was added to the culture at the final concentration of 0.1 mM, and cultivation was continued for an additional 8 h at 37  $^{\circ}$ C. Cells were harvested by centrifugation, suspended in extraction buffer (10 mM Tris-HCl buffer, pH 8.0, containing 0.5 mM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride), and sonicated. After sonication, the solution was centrifuged at 5,000  $\times$  g for 20 min, and the supernatant obtained was used as the crude enzyme solution. The purified recombinant CDase was obtained from the crude extracts by using a combination of chromatographies using DEAE-Sepharose FF, phenyl-Sepharose 6 FF, chelating-Sepharose FF, and TSKgel G3000SW (Tosoh, Japan) as described previously (21).

**Cloning, DNA Sequencing, and Expression of *Mycobacterium tuberculosis* CDase Homologue**—The *M. tuberculosis* cosmid MTC1376 was treated with *Not*I, and the digest was separated by 1.0% agarose gel electrophoresis. The 5-kbp fragment containing putative CDase homologue Rv0669c was cloned into *Not*I-digested pBluescript II SK. The recombinant plasmid was purified and designated pSMN5. PCR primers were designed using the start and stop codon regions of the gene, both of which contained a *Hind*III restriction site; sense oligonucleotide (5'-AGGAAAGCTTAATGCTTAGTGTAGGGCGCGCAT-3') and antisense oligonucleotide (5'-GCCGAAGCTTCAAACAACCGTGAACCTCGCGGT-3'). Using these primers and Pyrobest DNA polymerase (Takara Shuzo Co., Japan), a 1.9-kbp product was amplified with pSMN5 as the template. The amplified fragment was treated with *Hind*III, separated by electrophoresis in a 1.0% agarose gel, and cloned into *Hind*III-digested pTV118N vector. The recombinant plasmid was purified, and the insert was sequenced. The obtained plasmid was named pTMT4 and expressed in *E. coli* JM109 by the method described above.

**CDase Assay**—The activity of CDase was measured using C12-NBD-dodecanoylsphingosine (C12-NBD-ceramide) as the substrate by the method described previously (21). Briefly, the reaction mixture contained 550 pmol of C12-NBD-ceramide and an appropriate amount of the enzyme in 20  $\mu$ l of 25 mM Tris-HCl buffer, pH 8.5, containing 0.25% (w/v) of Triton X-100 and 2.5 mM CaCl<sub>2</sub>. Following incubation at 37  $^{\circ}$ C for 20 min, the reaction was terminated by heating in a boiling water bath for 5 min. The sample was evaporated, dissolved in 30  $\mu$ l of chloroform/methanol (2/1, v/v), and applied to a TLC plate which was developed with solvent I (chloroform/methanol/25% ammonia, 90/20/0.5, v/v). C12-NBD-ceramide was prepared by the method described by Tani *et al.* (25) using the reverse hydrolysis reaction (condensation) of sphingolipid ceramide *N*-deacylase (26). One enzyme unit of CDase was defined as the amount capable of catalyzing the release of 1  $\mu$ mol of C12-NBD-dodecanoic acid/min from the C12-NBD-ceramide under the conditions described above. A value of 10<sup>-3</sup> unit enzyme was expressed



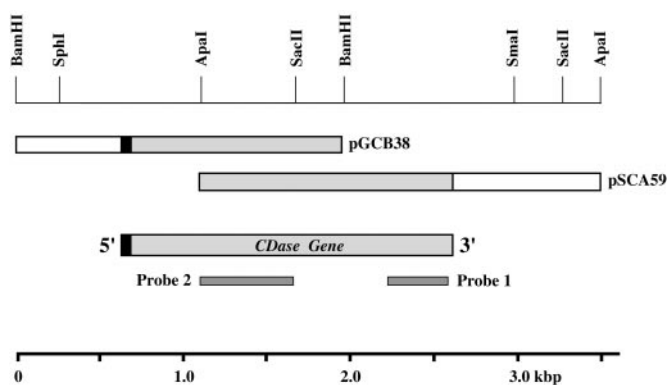


FIG. 1. Restriction map of two overlapping clones including the CDase gene. pSCA59, obtained by screening of the genomic library with Probe 1, and pGCB38, obtained by screening of the library with Probe 2, overlapped as shown in this figure. The black box corresponds to the putative signal sequence. Coding regions of CDase are indicated by shaded boxes.

as 1 milliunit in this study.

**Other Methods**—SDS-PAGE was performed by the method described by Laemmli (27). The nucleotide and amino acid sequences were evaluated using the DNASIS computer program developed by Hitachi Software Engineering (Japan).

## RESULTS

**Molecular Cloning of the Alkaline CDase Gene**—Five peptides were obtained from the purified *Pseudomonas* CDase after digestion with lysylendopeptidase and subjected to amino acid microsequencing. N-terminal amino acid sequence was also determined using the purified native enzyme. Finally, 64 amino acid residues of the CDase were determined (Table I). PCR was performed using one set of the primers (C89s, 5'-GAYGTSYTSARCARC-3'; and C91a, 5'-AAGCTYCASRASCCVTG-3') and *P. aeruginosa* strain AN17 genomic DNA as the template. A 359-bp PCR product was obtained (Probe 1) which contained DNA sequences encoding the peptide sequences of C-86, C-89, C-91, and C-121 (Table I). Southern blotting using <sup>32</sup>P-labeled Probe 1 showed a 2.4-kbp band in the genomic DNA digested with *Apa*I. A clone, pSCA59, containing a 2.4-kbp insert was isolated from the library enriched with the CDase gene. The nucleotide sequence of pSCA59 was determined, and a putative open reading frame was found. The amino acid sequences of C-86, C-89, C-91, and C-121 were found in the deduced amino acid sequences of the open reading frame, but N-terminal amino acid sequence was not. Therefore, we screened the genomic library to find the missing sequences using Probe 2 prepared from pSCA59. Southern blotting with <sup>32</sup>P-labeled Probe 2 of the *Bam*HI digest gave a 1.9-kbp band. A clone, pGCB38, containing a 1.9-kbp insert was isolated from the library enriched with the 5'-end of the CDase gene and sequenced. The deduced amino acid sequence contained the N-terminal amino acid sequence in the same frame. A partial sequence of the 5'-end of the CDase gene in pSCA59 was also found in pGCB38, indicating that sequences in both clones overlapped (Fig. 1).

**DNA and Deduced Amino Acid Sequences of the Alkaline CDase**—We cloned and sequenced 2,744 nucleotides of the two contiguous clones pSCA59 and pGCB38, including the coding regions of the CDase gene, and found an open reading frame from position 626–2,635 (Fig. 2). A hydrophobic motif composed of 24 amino acid residues was coded at nucleotides 626–697, starting with ATG. The presence of the hydrophobic motif was also clearly indicated by hydrophobicity plot analysis (Fig. 7A). This sequence motif, a putative secretion signal peptide, had a positively charged N terminus followed by a hydrophobic core and a string of polar residues (28). The finding of a signal

peptide sequence was in good agreement with the observation that *Pseudomonas* CDase was secreted into the culture medium. A possible Shine-Dalgarno ribosome binding sequence started 12 bases upstream of the ATG codon (Fig. 2). The open reading frame was 2,010 base pairs long with 670 codons. The gene encodes a signal sequence of 24 residues and a mature protein of 646 amino acid residues. The molecular weight of the CDase calculated from the deduced amino acid sequence was 70,767, in good agreement with that of the purified CDase estimated on SDS-PAGE ( $M_r = 70,100$ ). The predicted pI was at pH 5.73. All of the peptide sequences shown in Table I were found in the deduced amino acid sequence.

**Expression of the Alkaline CDase**—The expression plasmid pTCD11 was constructed by insertion of a fragment of the coding sequence with a Shine-Dalgarno ribosome binding sequence and a putative signal sequence into the *Hind*III site of plasmid pTV119N (Fig. 3). In pTCD11, transcription of recombinant genes is controlled by the promoter *plac* and can be induced by IPTG. *E. coli* JM109 cells transformed with pTCD11 were cultured in medium containing 0.1 mM IPTG and separated from the medium by centrifugation. The CDase activity of the cell lysate was assayed with C12-NBD-ceramide as the substrate. The expression of the CDase in *E. coli* increased with time after induction with IPTG and reached more than 30 units per liter of culture at 22 h (Fig. 4A), whereas strain AN17 of *P. aeruginosa* produced CDase at about 2 units per liter of culture (Fig. 4B). Mock transfectants carrying the plasmid pTV119N without the CDase gene showed no CDase activity, indicating that the observed enzyme activity was due entirely to expression of the cloned CDase gene. The recombinant enzyme, which could be transported to the periplasmic space in soluble form, was extracted by sonication and purified to homogeneity as described under "Experimental Procedures." The molecular weight of the recombinant CDase was estimated to be 70,100 by SDS-PAGE (Fig. 4C), which was consistent with that of the purified CDase from *P. aeruginosa* strain AN17 (21) and that deduced from the *Pseudomonas* CDase gene. The N-terminal amino acid sequence of the recombinant CDase was determined using a peptide sequencer and found to be identical to that of the native enzyme (DDLPHYRFGGL), indicating that the signal peptide of the recombinant CDase was cleaved off at the same site of the sequence of the native enzyme in host cells. Substrate specificity as well as optimum pH and metal-ion requirements of the recombinant CDase were identical to those of the native enzyme (data not shown).

**Cloning, Expression, and Functional Analysis of CDase Homologue from *Mycobacterium tuberculosis***—No significant sequence similarity was found in other known functional proteins including human acid CDase. However, we found sequences homologous to *Pseudomonas* CDase in the hypothetical proteins with unknown functions encoded in *M. tuberculosis*, *Dictpostelium discoideum*, and *Arabidopsis thaliana* using the GenBank™ data bank (accession numbers Z95972, U82513 and AB016885, respectively). Comparison of the CDase with these three sequences revealed identities of about 40%. Fig. 5 shows the alignment of *Pseudomonas* CDase and *Mycobacterium* hypothetical protein Rv0669c. In this study, we cloned the homologue Rv0669c from the cosmid MTCI376 of *M. tuberculosis* using the method described under "Experimental Procedures." The expression plasmid pTMT4 carrying the cloned Rv0669c was thus constructed and used to transform *E. coli* JM109. Interestingly, cell lysates of the transformed *E. coli* carrying Rv0669c hydrolyzed C12-NBD-ceramide to produce NBD-dodecanoic acid, whereas mock transfectants did not (Fig. 6A). This result clearly indicated that the CDase homologue Rv0669c encoded the CDase. It is particularly interesting to

GGATCCTCTCGGCATCTGGATGATGGCGGTGCGAGTACATCGACTATCCGGCGGACAACCAAGCTCGGCTGGAAGAGATGCT	85
CGCTTGGCTGCGCAGCAAGCGCTGGCGGTGATCGGGTTTCGGCGGGGTGACCTACCTGGCGCTGCTGATCCCGCTGTCAACCTGGTCAT	175
GATGCCCGCCCGCTCGCCGGCCACCTGTTTCGGCTCGCGAGGAGGCGAGAGGCGCTGGTGAATAAGCATGCGCCGGCTGCC	265
GCTGTCGGCATTTGTCAGCGGGCGTCAAGCCCTGACAGCGCGCTCGGGCACACTACGAATGCCCTCGGGAGCGCTCGGCGAGGCGCA	355
GCCGAGACCTGCGCGCCCTTGCAGACCGCTCGACGCTGCGGAACCGACTGCGCCGGTGCCTCCCGACGAGCGCGCTTTTPTTA	445
CCCGCTGCTTGCCTGCTCATGAGATGGCGCGCTCACCCCTTCTGTCGCTACGCCCCCTCGACTCCACACCTGGCACTAC	535
AGTGGCAACAGCGAAGCAGGCTCGGCGCGCTTTTCGATGACCCGCTCCCGAGCCGCGCGGAAACAAGAGGGGTGCGC	625
ATGTCACGTTCCGATTCACCGCGCTTTCCTGCTCGCTCTGCTGCGCTCTCCATGCTGCGAGGCGGACGACCTGCCCTACCGC	715
<u>M S R S A F T A L L L S C V L L A L S M P A R A</u> D D L P Y R	6
-24 -1 +1 C-118(S-90)	
TTCCGCTTGGCAAGGCGACATCACCGCGAAGTCGGCATGATGGTTACTCTCCCTCGACAGAAGACCGCGCGCAT	805
<u>F G L G K A D I T G E A A E V G M M G Y S S L E Q K T A G I</u>	36
CACATGCGCGATGGGGCGCTTTCGTGATCGAGGAAGCGCGCAGCGAGCTCGCCTGGTACTAGTCAACACCGACCTGGGGATGACC	895
H M R Q W A R A F V I E E A A S G R R L V Y V N T D L G M T	66
TTCCAGGCGTGCACCTGAAGTCTTGGCGCGCTCAAGGCGAAGTACCCCGGTGTCTACGACGANNACAACGATGCTCGCCGCCAC	985
F Q A V H L K V L A R L K A K Y P G V Y D E N N V M L A A T	96
CACACCACTCGGTCGGCGCGCTTCCCACTACGCGATGACAACTGTCCGTGCTCGGCTTCCAGGAAAAGACCTTCAACGCCATC	1075
H T H S G P G G F S H Y A M Y N L S V L G F P Q E K T F N A I	126
GTCGACGCGATCTCCGCTCCATCGAGCGGCGCCAGGCGAGTTCAGCGCGCGCGCTGTTCTACGCGCGCGGAGCTGCGCAACGCC	1165
V D G I V R S I E R A Q A R L Q P G R L F Y G A S G E L R L A	156
AGCCGCAACCGTTCGCTGCTGCGCACTGAAGAATCCGGCACTCGCGCGCTACGAGGATGCGACCGCGAGATGAGCTGCTCGCAGC	1255
S R N R L L S H L L K N P D I A G Y E D G I D P Q M S V P L S	186
TTCCGACGCGCAACCGCGAGCTGGCGCGCGCATGATGGTTCCCGGTGCACAGCACCTCGATGACCAACCGCAATCACCTGATCTCC	1345
F V D A N G E L A G A I S W F P V H S T S M T N A N H L I S	216
CCGGACACCAAGGCTACCGCTCTCTATCAGTGGAGCAGCAGCTCAGCGCAAGCGGTTTCTCGCCGCTTCCCGCAGACCAATGCC	1435
P D N K G Y A S Y H W E H D V S R K S G F V A A F A Q T N A	246
GCAACCTGCGCCCAACTGAACCTGAAGCCGCGCTCGGCTTCCGCTTCCGACACCGCGAGATGCGTGTGCGCCAA	1525
G N L S P N L N L K P G S G P F D N E F D N T R E I G T L G R Q	276
TTCCCAAGCGCTACGAGATCGCGCGCGAGCGCCAGGAGGAAGTGTCCGCGAATGGATTCCGCTTCCGTTCTCGCTGACTTCAACCGC	1615
F A K A Y E I A G Q A Q E E V L G E L D S R F R F V D F T R	306
CTGCGCTACCGCCCGAGTTCAACCGCGCGAGTGTGACCGCGCGCATGTCAGCGCGCGCATGTCGCGCGCGCGCGCGCGCGCGAA	1705
L P I R P E F T D G Q P R Q L C T A A I G T S L A A G S T E	336
GACGCTCAGCGCGCTGGCGGTGAGGAAAGCAACAATCCGTTCTCTCGCGCTTCCGCGGTGCTCAGCGCGTGGCCCGCAGGAA	1795
D G P G P L G L E E G N N P F L S A L G G L L T G V P P Q E	366
CTGTGCAATGCCAGCGGAAAGCAACTCTCGCCGACACCGCAACCAAGAACTACCCCTGGACCGCGAGGCTGCTCGCGATCCAG	1885
L V Q C Q A E K T I L A D T G N K K P Y P W T P T V L P I Q	396
ATGTTCCGATCGCGCGAGTTGAACTGCTCG	1975
M F R I G Q L E L L G A P A E F T V M A G V R I R R A V Q A	426
GCCAGCAAGCGCGGCTTCCGCGATGTTGCTTCAATGGCTACCGAATGCCTATGCCAGTACGTCACCAACCGCGAGGAATACGCC	2065
A S E A A G I R H V V F N G Y A N A Y A S Y T V R E E Y A	456
GCCAGGAATACGAAGCGCGCTCGACCTCTACCGCCCTGGACCGCGCGCTACCAGCAGTTGTTGCTGACATGGCGGCGCGCTG	2115
A Q E Y E G G S T L Y G P W T Q A A Y Q Q L F V D M A V L A	486
CCCGACCGCTGCGCGTGGAACTCGCGGATAGCG	2245
R E R L P V E T S A I A P D L S C C Q M N F Q T G V V A D D	516
CCCTATATCGCAAGTCTTCCGCGAGCTGTTGCAACAACCGAGGAAAGTTATCGCATCGCGGACAGGCTGCGCTTCCGCTGACC	2335
P Y I G K S F G D V L Q Q P R E S Y R I G D K V T V A P V T	546
GGACATCGGAAGTACTTCCGCGCGAGGACTTTCCTGGAAGTGGTGAATATCGCAAGGATGGCAACAGAGCGCGCTGACCGCT	2425
<u>G H P K N D L R T E K T F L E V V N I G K D G K Q T P V T</u>	576
C-121	
GCCACCGATAAGTACTGGGATACCAATACCGCTGGGAGAGAGTGGGTATATCTGCTCGAAAGCGACTATCAGTGGCTTCCATCCACCA	2515
A T D N D W D T Q Y R W E R V G I S A S K A T I S W S I P P	606
GGACCGAGCGCGCGCTTACTACACTAGGCACTATGGCAACCGGAAAGTCTTGGACCGAGAAGTACGCGGAAATCGCGCGCTGACC	2605
G T E P G H Y I R H Y G N A K N F W T Q K I S E I G G S T	636
C-91	
CGCTCTCGAGGTGCTCGCGCACTCCCTAGCGCGCGCTCCAGCCAGGTTTCGAGATTCGCGAGCGCAACTTATGACGATGAAAGT	2695
<u>R S F E V L G T T P *</u>	646
GTCAAATAAATGTGATTTAAACACATGAACAAGTACCTTTTCATTCA	2744

FIG. 2. DNA and deduced amino acid sequences of the CDase gene and flanking sequences. The deduced amino acid sequence of the CDase is shown as 1-letter symbols below the nucleotide sequence, and amino acid residues are numbered beginning with the N-terminal amino acid (asparatic acid). Amino acids determined by peptide sequencing are underlined; the possible Shine-Dalgarno sequence is double underlined; the translation termination codon is denoted by an asterisk. Numbers to the right of the sequence correspond to amino acids (lower) and nucleotides (upper).

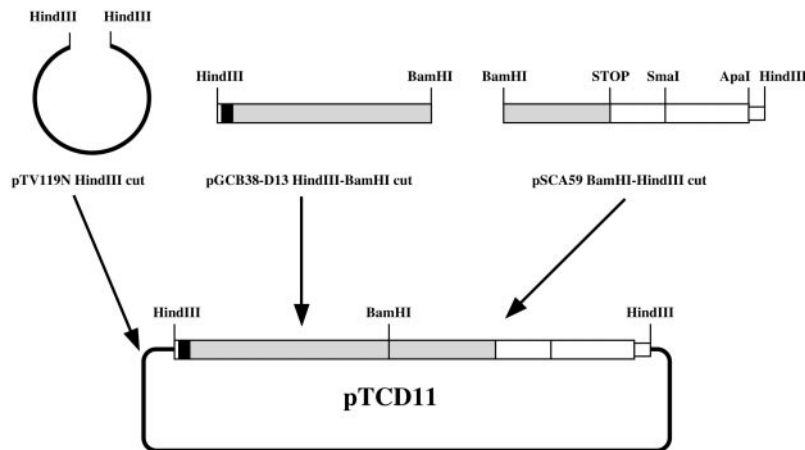


FIG. 3. Construction of the CDase expression plasmid. The CDase expression plasmid was constructed by the method described under "Experimental Procedures." The black box corresponds to the putative signal sequence. Coding regions of CDase are indicated by shaded boxes.

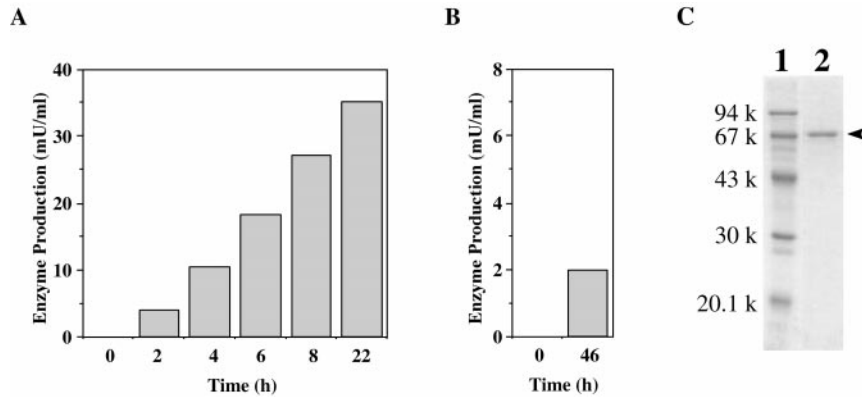
note that the recombinant protein of *M. tuberculosis* functioned as a CDase at alkaline pH with an optimum at pH 8.5 (Fig. 6B). The profile for the pH optimum of *Mycobacterium* CDase was found to be very close to that of *Pseudomonas* CDase (21). On the other hand, metal ion dependence of *Mycobacterium* CDase was completely different from that of *Pseudomonas* enzyme (Fig. 6C). *Pseudomonas* CDase was activated by  $Ca^{2+}$  and completely inhibited by EDTA at 2 mM, whereas *Mycobacterium* enzyme was strongly inhibited by  $Ca^{2+}$  and not affected

by EDTA at the same concentration.  $Mg^{2+}$  was found to inhibit the activities of both CDases at 2 mM.

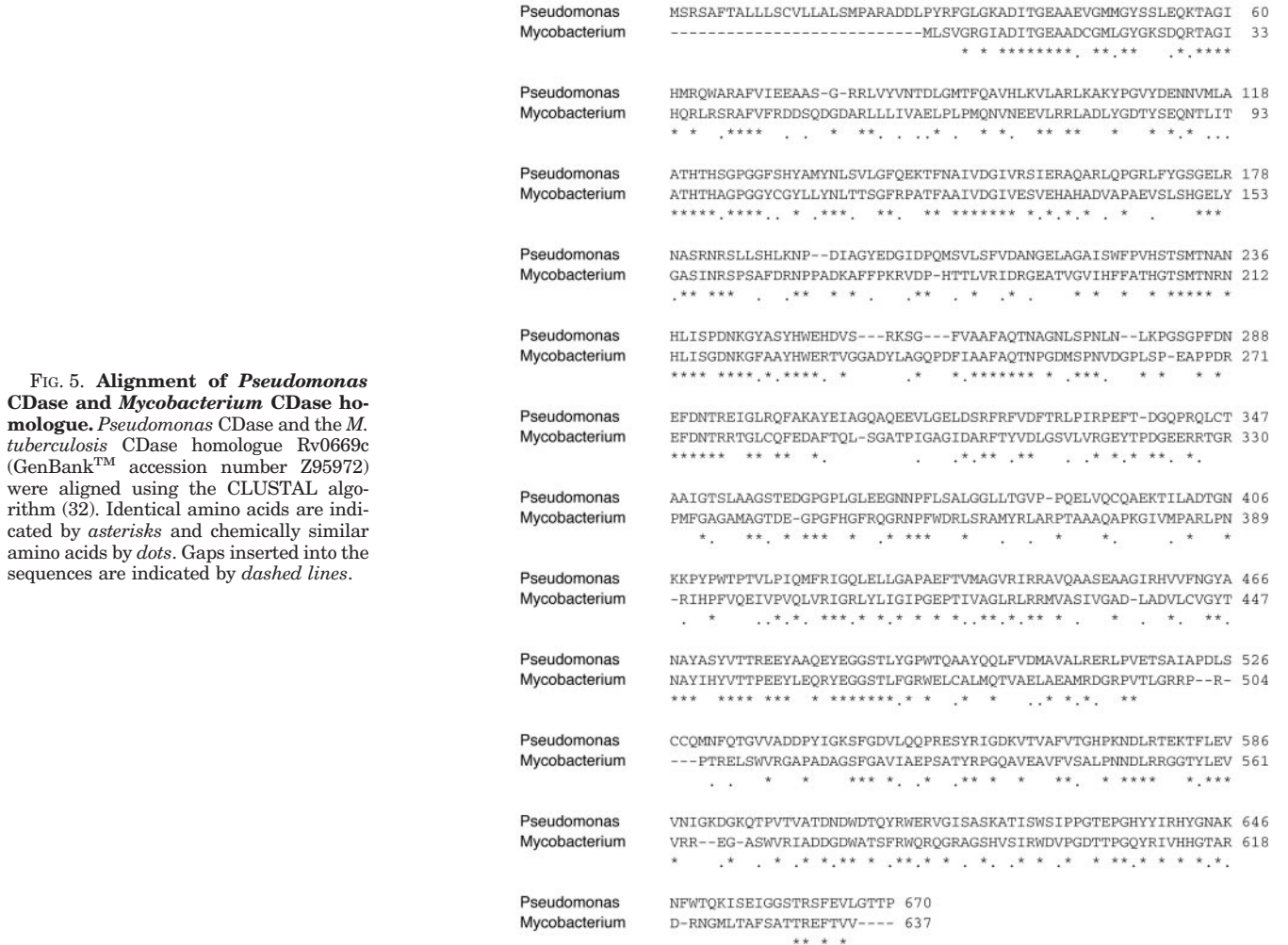
In contrast to the *Pseudomonas* enzyme, *Mycobacterium* CDase lacked the putative signal sequence (Fig. 5), and this was confirmed by hydrophobicity plot analysis (Fig. 7B).

#### DISCUSSION

In the present study, we cloned, sequenced, and expressed the gene encoding CDase which exhibited an alkaline pH opti-



**FIG. 4. Production of CDase by *E. coli* JM109 containing pTCD11 (A), *P. aeruginosa* strain AN17 (B), and SDS-PAGE of the purified recombinant CDase (C).** A, time course for production of recombinant CDase after induction by IPTG. *E. coli* JM109 cells transformed with pTCD11 were cultured by the method described under “Experimental Procedures.” After addition of IPTG, culture was continued at 37 °C for 22 h. At the indicated times, CDase activity of the cell lysate was determined. B, production of CDase by *P. aeruginosa* strain AN17. Cells were transferred to PY medium (0.5% polypepton, 0.1% yeast extract, 0.5% NaCl, pH 7.2) containing sphingomyelin at a concentration of 0.01%. Following incubation at 37 °C for 46 h. CDase activity in the culture supernatant was determined as described under “Experimental Procedures.” C, the final preparation of recombinant CDase was analyzed by SDS-PAGE under reducing conditions. Proteins were stained with Gelcode® Blue Stain Reagent (Pierce). Lane 1, standard proteins; lane 2, purified recombinant CDase. Standard weights in parentheses were: phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100).

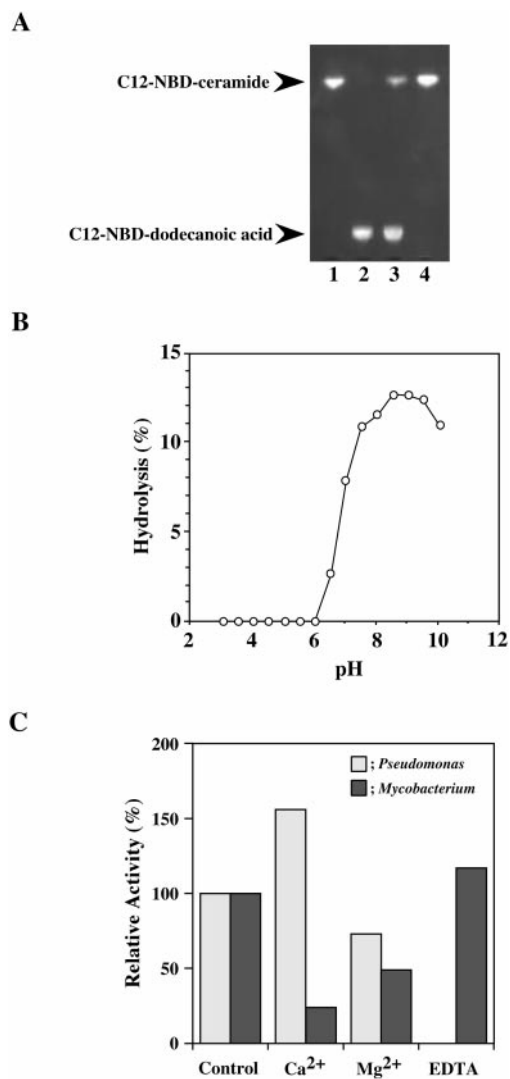


**FIG. 5. Alignment of *Pseudomonas* CDase and *Mycobacterium* CDase homologue.** *Pseudomonas* CDase and the *M. tuberculosis* CDase homologue Rv0669c (GenBank™ accession number Z95972) were aligned using the CLUSTAL algorithm (32). Identical amino acids are indicated by asterisks and chemically similar amino acids by dots. Gaps inserted into the sequences are indicated by dashed lines.

mum from *P. aeruginosa* strain AN17. This is the first report for the gene cloning of not only alkaline but also prokaryotic CDase. The deduced amino acid sequence has no significant sequence similarity with other known proteins including cloned human acid CDase (6). However, sequences homologous to the *Pseudomonas* CDase were found in the hypothetical

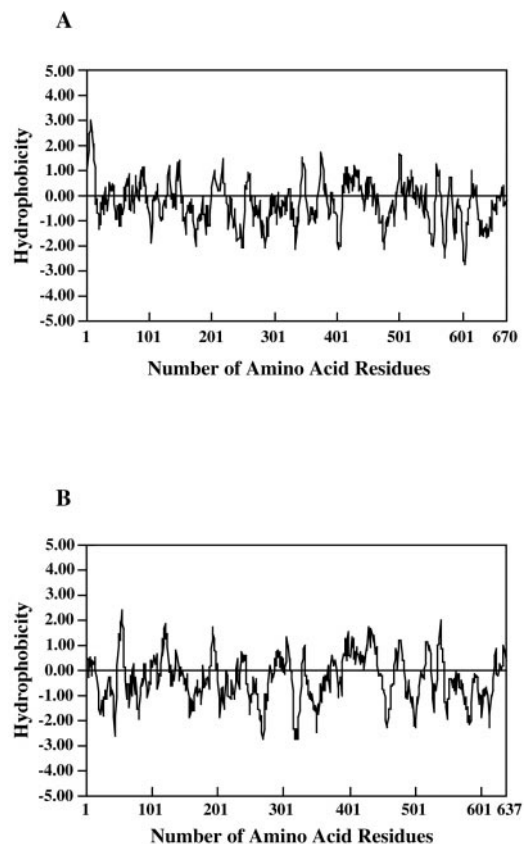
proteins in *M. tuberculosis*, *D. discoideum*, and *A. thaliana*. These proteins showed about 40% amino acid identity to *Pseudomonas* CDase. We cloned and expressed the gene of *M. tuberculosis* Rv0669c in *E. coli* JM109 in this study. The recombinant CDases from *M. tuberculosis*, a Gram-positive tubercle bacillus, and *P. aeruginosa*, a Gram-negative opportu-





**FIG. 6. Expression of *Mycobacterium* CDase homologue in *E. coli* JM109 cells (A), pH-dependence of *Mycobacterium* CDase (B), cation-dependence of the recombinant CDases (C).** Rv0069c was expressed in *E. coli* JM109 by the method described under "Experimental Procedures." A, the reaction mixture contained 100 pmol C12-NBD-ceramide and an appropriate amount of the enzyme in 20  $\mu$ l of 25 mM Tris-HCl buffer, pH 8.5, containing 0.25% (w/v) Triton X-100. Following incubation at 37  $^{\circ}$ C for 1 h, the samples were evaporated, dissolved in 15  $\mu$ l of chloroform/methanol (2/1, v/v), and applied to a TLC plate which was developed with solvent I. Lane 1, C12-NBD-ceramide; lane 2, C12-NBD-dodecanoic acid; lane 3, cell extract of *E. coli* JM109 containing pTMT4 with C12-NBD-ceramide; lane 4, cell extract of mock (pTV118N) transfectant with C12-NBD-ceramide. B, CDase activity was determined by the method described under "Experimental Procedures." In this experiment, 150 mM GTA buffer (50 mM 3,3-dimethylglutaric acid, 50 mM Tris(hydroxymethyl) aminomethane, and 50 mM 2-amino-2-methyl-1,3-propanediol) at the indicated pH was used. C, reaction mixtures containing CDase, 2 mM of each cation, and 100 pmol of C12-NBD-ceramide in 20  $\mu$ l of 25 mM Tris-HCl buffer, pH 8.5, containing 0.25% (w/v) Triton X-100 were incubated at 37  $^{\circ}$ C for 20 min. The hydrolysis of C12-NBD-ceramide was determined by the method described under "Experimental Procedures." The amounts of enzyme used were 5.4 microunits for recombinant *Pseudomonas* CDase, and 4.6 microunits for recombinant *Mycobacterium* CDase. Values are the means for duplicate determinations.

nistic pathogen, showed alkaline pH optima although the low similarity of the amino acid sequences of both CDases suggested that some of the properties of the enzymes might differ from each other. In fact, *P. aeruginosa* CDase required Ca<sup>2+</sup> for expression of its activity (21), whereas the *M. tuberculosis* enzyme did not (Fig. 6C). Interestingly, Rv0669c lacked the putative signal peptide sequences (Fig. 7B), suggesting that



**FIG. 7. Hydropathy plots for *Pseudomonas* (A) and *Mycobacterium* (B) CDases.** The deduced amino acid sequence of two bacterial CDases were analyzed by the method of Kyte and Doolittle (33) for hydrophobicity plotting. Amino acid residues are numbered beginning with the first methionine.

*Mycobacterium* CDase resides in the cytosol. This is in contrast to the *Pseudomonas* CDase, which is released into the culture medium. The CDase secreted by *P. aeruginosa* may contribute to its pathogenicity as a kind of exotoxin, similarly to that of phospholipase C, elastase, and alkaline proteases (29).

Over the last few decades, the incidence of tuberculosis, a chronic infectious disease caused by *M. tuberculosis*, has been increasing worldwide and is becoming a serious social problem. Analysis of the complete genome sequence of *M. tuberculosis* has revealed that the bacillus clearly differs from other bacteria in that a very large proportion of its genes encoded enzymes involved in lipogenesis and lipolysis (30). The enzyme encoded by the homologue gene in *M. tuberculosis*, which was identified for the first time in this study, might function by decomposing the ceramides of host cells, although the pathogenic significance of *Mycobacterium* CDase remains to be clarified.

CDase of *P. aeruginosa* strain AN17 is a powerful tool for studying the structure and function of ceramide, but the level of production of the enzyme by this strain is low. Cloning and expression of the *Pseudomonas* CDase gene resolved the difficulty in the preparation of large amounts of CDase, i.e. the production of CDase by transformed *E. coli* cells carrying the CDase gene reached 30 units/liter of culture, which is 15-fold higher than that by the wild-type strain of *P. aeruginosa* strain AN17 (Fig. 4, A and B).

Zhang *et al.* (31) expressed the bacterial sphingomyelinase gene in Molt-4 leukemia cells and observed an increase of intracellular sphingomyelin hydrolysis, resulting in the induction of apoptosis possibly because of an increase in ceramide level in transformed cells. Recently, we expressed the cloned CDase gene in CHO cells as a fusion protein with green fluo-

rescent protein.<sup>2</sup> The CDase gene cloned in this study will become a useful molecular probe for elucidation of sphingosine-mediated signal transduction.

**Acknowledgments**—We thank Mr. H. Izu, Biotechnology Laboratories, Takara Shuzo Co, for technical advice. We also thank Dr. S. T. Cole, Institut Pasteur, France for providing the *M. tuberculosis* cosmid MTCI376.

## REFERENCES

- Gatt, S. (1963) *J. Biol. Chem.* **238**, 3131–3133
- Gatt, S. (1966) *J. Biol. Chem.* **241**, 3724–3730
- Hassler, D. F., and Bell, R. M. (1993) *Adv. Lipid Res.* **26**, 49–57
- Sugita, M., Dulaney, J. T., and Moser, H. W. (1972) *Science* **178**, 1100–1102
- Bernardo, K., Hurwitz, R., Zenk, T., Desnick, R. J., Ferlinz, K., Schuchman, E. H., and Sandhoff, K. (1995) *J. Biol. Chem.* **270**, 11098–11102
- Koch, J., Gärtner, S., Li, C.-M., Quintern, L. E., Bernardo, K., Levran, O., Schnabel, D., Desnick, R. J., Schuchman, E. H., and Sandhoff, K. (1996) *J. Biol. Chem.* **271**, 33110–33115
- Li, C.-M., Hong, S.-B., Kopal, G., He, X., Linke, T., Hou, W.-S., Koch, J., Gatt, S., Sandhoff, K., and Schuchman, E. H. (1998) *Genomics* **50**, 267–274
- Coroneos, E., Martinez, M., McKenna, S., and Kester, M. (1995) *J. Biol. Chem.* **270**, 23305–23309
- Bielawska, A., Greenberg, M. S., Perry, D., Jayadev, S., Shayman, J. A., McKay, C., and Hannun, Y. A. (1996) *J. Biol. Chem.* **271**, 12646–12654
- Nikolova-Karakashian, M., Morgan, E. T., Alexander, C., Liotta, D. C., and Merrill, A. H., Jr. (1997) *J. Biol. Chem.* **272**, 18718–18724
- Hannun, Y. A., Loomis, C. R., Merrill, A. H., Jr., and Bell, R. M. (1986) *J. Biol. Chem.* **261**, 12604–12609
- Oishi, K., Zheng, B., and Kuo, J. F. (1990) *J. Biol. Chem.* **265**, 70–75
- Pushkareva, M. Yu., Khan, W. A., Alessenko, A. V., Sahyoun, N., and Hannun, Y. A. (1992) *J. Biol. Chem.* **267**, 15246–15251
- Stoffel, W., Bauer, E., and Stahl, J. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 61–74
- Spiegel, S., and Merrill, A. H., Jr. (1996) *FASEB J.* **10**, 1388–1397
- Spiegel, S., Foster, D., and Kolesnik, R. N. (1996) *Curr. Opin. Cell Biol.* **8**, 159–167
- zu Heringdorf, D. M., van Koppen, C. J., and Jakobs, K. H. (1997) *FEBS Lett.* **410**, 34–38
- Lee, M.-J., Van Brocklyn, J. R., Thangada, S., Liu, C. H., Hand, A. R., Menzeleev, R., Spiegel, S., and Hla, T. (1998) *Science* **279**, 1552–1555
- Okamoto, H., Takuwa, N., Gonda, K., Okazaki, H., Chang, K., Yatomi, Y., Shigematsu, H., and Takuwa, Y. (1998) *J. Biol. Chem.* **273**, 27104–27110
- Merrill, A. H., Jr., and Wang, E. (1986) *J. Biol. Chem.* **261**, 3764–3769
- Okino, N., Tani, M., Imayama, S., and Ito, M. (1998) *J. Biol. Chem.* **273**, 14368–14373
- Dainese, H. P., Quadroni, M., Staudenmann, W., and James, P. (1997) *Eur. J. Biochem.* **264**, 336–343
- Sambrook, H., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Saito, H., and Miura, K. (1963) *Biochim. Biophys. Acta* **72**, 619–629
- Tani, M., Kita, K., Komori, H., Nakagawa, T., and Ito, M. (1998) *Anal. Biochem.* **263**, 183–188
- Ito, M., Kurita, T., and Kita, K. (1995) *J. Biol. Chem.* **270**, 24370–24374
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Abrahamsen, L., Moks, T., Nilsson, B., Hellman, U., and Uhlen, M. (1985) *EMBO J.* **4**, 3901–3906
- Bodey, G. P., Bolivar, R., Fainstein, V., and Jadeja, L. (1983) *Rev. Infect. Dis.* **5**, 279–313
- Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry III, C. E., et al. (1998) *Nature* **393**, 537–544
- Zhang, P., Liu, B., Jenkins, G. M., Hannun, Y. A., and Obeid, L. M. (1997) *J. Biol. Chem.* **272**, 9609–9612
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22**, 4673–4680
- Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132

<sup>2</sup> N. Okino, K. Nara, and M. Ito, unpublished results.

---

**CARBOHYDRATES, LIPIDS, AND  
OTHER NATURAL PRODUCTS:  
Molecular Cloning, Sequencing, and  
Expression of the Gene Encoding Alkaline  
Ceramidease from *Pseudomonas aeruginosa*  
: CLONING OF A CERAMIDASE  
HOMOLOGUE FROM  
MYCOBACTERIUM TUBERCULOSIS**

Nozomu Okino, Sachiyo Ichinose, Akira  
Omori, Shuhei Imayama, Takashi Nakamura  
and Makoto Ito  
*J. Biol. Chem.* 1999, 274:36616-36622.  
doi: 10.1074/jbc.274.51.36616

---

Access the most updated version of this article at <http://www.jbc.org/content/274/51/36616>

Find articles, minireviews, Reflections and Classics on similar topics on the [JBC Affinity Sites](#).

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 32 references, 18 of which can be accessed free at  
<http://www.jbc.org/content/274/51/36616.full.html#ref-list-1>