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Cloning and Expression of Gene Encoding a Novel Endoglycoceramidase of *Rhodococcus* sp. Strain C9[1]

Keishi Sakaguchi,* Nozomu Okino,* Noriyuki Sueyoshi,* Hiroyuki Izu,† and Makoto Ito*,[2]

*Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, School Kyushu University, 6-10-1, Hakozaki, Higashi-ku, Fukuoka 812-8581, and †Biotechnology Research Laboratories, Takara Shuzo Co., Shiga

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Endoglycoceramidase (EGCase) is an enzyme capable of cleaving the glycosidic linkage between oligosaccharides and ceramides of various glycosphingolipids. We previously reported that the Asn-Glu-Pro (NEP) sequence is part of the active site of EGCase of *Rhodococcus* sp. strain M-777. This paper describes the molecular cloning of a new EGCase gene utilizing the NEP sequence from the genomic library of *Rhodococcus* sp. strain C9, which was clearly distinguishable from M-777 by 16S rDNA analysis. C9 EGCase possessed an open reading frame of 1,446 bp encoding 482 amino acids, and showed 78% and 76% identity to M-777 EGCase II at the nucleotide and amino acid levels, respectively. Interestingly, C9 EGCase showed the different specificity to the M-777 enzyme: it hydrolyzed b-series gangliotetraosylceramides more slowly than the M-777 enzyme, whereas both enzymes hydrolyzed a-series gangliosides and neutral glycosphingolipids to the same extent.

Key words: endoglycoceramidase, gangliosides, glycosphingolipids, homology cloning, NEP sequence, substrate specificity.

Glycosphingolipids (GSLs), amphipathic compounds consisting of oligosaccharide and ceramide (Cer) moieties, have been reported to function as tumor antigens, receptors for microbes and their toxins, and possible modulators of various cellular activities (*1*). Recently, GSLs were found to be enriched with cholesterol and GPI-anchor proteins to form microdomains on the plasma membrane of vertebrates (*2*). Endoglycoceramidase (EGCase: EC 3.2.1.123; also called Cer glycanase) cleaves the glycosidic linkage between oligosaccharides and Cers of various GSLs. The enzyme has been found in actinomycetes (*3*), bacteria (*4*), leechs (*5*), earthworms (*6*), and clams (*7*). Three isoforms of EGCase (EGCase I, II, and III) differing in molecular weight, pI, and substrate specificity, were found in *Rhodococcus* sp. M-777 (*8*). Recently, the gene encoding EGCase II of *Rhodococcus* sp. M-777 was cloned (*9*). The amino acid sequence of EGCase II was in part homologous to that of the active site region of cellulase (endo-1,4-β-glucanase: EC3.2.1.4), which belongs to family A of cellulases. We found that the Asn-Glu-Pro (NEP) sequence, the catalytic domain of family A cellulases, was also conserved in EGCase II and demonstrated that the NEP sequence was integral for expression of EGCase II activity (*10*).

We report here the cloning and expression of a new EGCase gene of *Rhodococcus* sp. C9 using the NEP sequence. Interestingly, the specificity of C9 EGCase was different from that of M-777 EGCase II: the C9 enzyme hydrolyzed b-series gangliosides such as GD1b, GT1b, and GQ1b more slowly than the M-777 enzyme.

MATERIALS AND METHODS

Materials—Escherichia coli strain JM109, and plasmid pTV118N and pTV119N were obtained from Takara Shuzo (Shiga). Precoated Silica Gel 60 TLC plates were purchased from Merck (Germany). Gangliosides GD1b was from Waco Chemical (Osaka). GM1 was prepared from bovine crude gangliosides using sialidase-producing bacteria as a microbial biocatalyst as described in Ref. *11*. Asialo-GM1 was prepared from GM1 as described elsewhere (*8*). Neolactotetra-osylCer (nLc_4Cer) and neolactohexaosylCer (nLc_6Cer) were kindly donated by Dr. H. Higashi, Mitubishi Kagaku Institute of Life Sciences (Japan).

Isolation and Identification of Rhodococcus sp. Strain C9—EGCase-producing bacteria were isolated by an enrichment culture method using synthetic medium A (0.05% NH_4Cl, 0.05% K_2HPO_4, 0.2% NaCl, and 0.05% TDC, pH 7.5) containing 0.1% GM1 as a sole source of carbon. Samples (soil, pond water, *etc.*) were suspended in 100 μl of synthetic medium and incubated at 30°C for 2 days. After incubation, 10-μl portions of cultures were evaporated with a Speed Vac concentrator, and the residues were dissolved in 15 μl of chloroform/methanol (2:1, v/v), and applied to TLC plates, which were then developed with chloroform/methanol/0.02% $CaCl_2$ (5:4:1, v/v). GM1-oligosaccharide generated by the action of EGCase was visualized by use of orcinol-H_2SO_4 reagent. The EGCase-positive cultures show-

[1] The nucleotide sequence reported in this paper has been submitted to the GenBank™ EBI Data Bank

[2] To whom correspondence should be addressed. Fax: +81-92-642-2900 or 2907, E-mail: makotoi@agr.kyushu-u.ac.jp

Abbreviations. Cer, ceramide; EGCase, endoglycoceramidase, GSL(s), glycosphingolipid(s); kbp, kilobase pair(s), IPTG, isopropyl-thio-β-D-galactopyranoside; PCR, polymerase chain reaction, NBD, 4-nitrobenzo-2-oxa-1,3-diazole, TLC, thin-layer chromatography. The structures of GSLs are shown in Table II.

ing the generation of GM1-oligosaccharide were selected, and EGCase-producing bacteria were isolated from them by single colony selection using Trypto-Soya agar plates (Nissui Seiyaku, Tokyo). The new EGCase-producing bacteriaum designated strain C9 was isolated from soil. The identification of C9 was mainly conducted according to the 8th edition of *Bergey's Manual of Determinative Bacteriology* (*12*). Quinone was analyzed as described elsewhere (*13*). C9 found to be a non-motile, Gram-positive, oxidase-positive, catalase-negative, aerobic actinomycetes. C9 was changed from rod-shaped to spherical during cultivation. Its G + C content was 69%. The quinone of C9 was determined to be MK-8(H_2). Judging from these results, C9 was assigned to the genus *Rhodococcus*.

Phylogenetic Analysis—The 16S rDNA was amplified using universal primers p27f (5′-AGA GTT TGA TCM TGG CTC AG-3′; positions 8-27; *Escherichia coli* numbering [*14*]) and p1492r (5′-GGC TAC CTT GTT ACG ACT T-3′). PCR products were purified from 1.0% agarose gel and were sequenced directly. The following sequencing primers were used (*15*):

r1L (5′-GTA TTA CCG CGG CTG CTG G-3′; position 536–518),
r2L1 (5′-CAT CGT TTA CGG CGT GGA C-3′; position 821–803),
r2L2 (5′-GAC TAC CAG GGT ATC TAA-3′; position 805–786),
r3L (5′-TTG CGC TCG TTG CGG GAC T-3′; position 1111–1093),
r4L (5′-ACG GGC GGT GTG TAC AAG-3′; position 1406–1389),
rE1L (5′-GTA GGA GTC TGG ACC GTG T-3′; position 345–327),
f1L (5′-GAG TTT GAT CCT GGC TCA G-3′; position 9–27),
f2L (5′-CCA GCA GCG GCG GTA ATA G-3′; position 518–536),
f3L (5′-GTC CCG CAA CGA GCG CAA C-3′; position 1094–1112).

The nucleotide sequences were aligned and phylogenetic relationships were analyzed using CLUSTAL W (*16*), with other 16S rDNA sequences obtained from the Ribosomal Database Project II (*17*).

Molecular Cloning—For cloning of the new EGCase gene of C9, PCR primers were designed based on the sequence of M-777 EGCase II gene (*9*). These primers were designated NEP-primers because they contained the NEP sequence, which is the putative active site of EGCase (*10*). NEP-primers used were as follows: U1 (5′-CCT GCG CGG GTT CAA CAC G-3′; complementary to the M-777 EGCase II gene sequence at position 165-183), and L1 (5′-AGG GAT CCT CCG AAC GGC TCG-3′; position 716–696). PCR was performed in 50 μl of a reaction mixture containing each NEP-primer at 0.5 μM, 0.5 μg of template genomic DNA, 0.2 mM dNTPs (dATP, dGTP, dCTP, and dTTP each at 0.5 μM), 2 mM $MgCl_2$, and 2.5 mU of amplitaq Gold (PE Biosystems, USA). PCR products were extracted from 1% agarose gel, and TA cloning was achieved using pGEM-T-Easy vector kit system I (Promega, USA). The resulting plasmid was designated pGEMU1-L1. Genomic DNA of C9 (10 μg) was digested with *Pst*I, then fractionated by 0.8% agarose gel electrophoresis as described elsewhere (*18*). DNA was transferred from agarose gels to nylon membranes (Hybond N^+, Amersham Pharmacia Biotech, UK) as described by the manufacturer. A DNA probe was prepared by digestion of pGEMU1-L1 with *Eco*RI and labeled with [α-^{32}P]-dCTP using a Ready-To-Go™ DNA labeling kit (Amersham Pharmacia Biotech, UK). Hybridization was performed in 0.5 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 7% SDS at 65°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°C and exposed on an imaging plate, which was then examined after several hours using a BAS1000 imaging analyzer (Fuji Film, Tokyo). Southern blot of *Pst*I digest showed that the 4.2-kbp fragment contained the C9 EGCase gene. To obtain the fragment, 10 μg of genomic DNA was digested with *Pst*I, and the restriction fragments were fractionated by 0.8% agarose gel electrophoresis. Fragments (4.2 kbp) were extracted from the gel and inserted into the *Pst*I site of pBluescript II SK (Toyobo, Osaka). *E. coli* JM109 was transformed with the recombinant plasmids and used as a DNA library enriched with C9 EGCase gene. Colony hybridization was performed by the standard procedure (*18*) using the probe described above, one clone was selected, and the plasmid was designated pBC82 .

DNA Sequencing and Sequence Analysis—The nucleotides were sequenced with a BigDye Terminator Ready Reaction Kit (PE Biosystems, USA). Sequencing gel electrophoresis was performed, and nucleotide sequences were automatically determined by using a DNA sequencer (Applied Biosystems, model 377). Computer analysis including comparison of DNA sequences was performed using DNASIS (Hitachi Software Engineering, Kanagawa).

Construction of Expression Vector with C9 EGCase Gene—PCR primers for introducing an *Nco*I site at the 5′ end of the PCR product were designed as follows: S1N (5-CCA TGG GGA GTG ACT CGA GCG-3), A1B (5-ACT CTC CGA GGA TCA CCG GTG-3). S1N contained a *Nco*I restriction site (underlined). PCR was performed with these primers using pBC82 as the template, the product was gel purified, and TA cloning was performed. By digestion of resultant plasmids with restriction endonuclease, *Nco*I–*Bam*HI fragments containing the N-terminal region of the C9 EGCase gene were prepared, and these were inserted into *Nco*I/*Bam*HI site of pTV119N with the *Bam*HI–*Bam*HI fragment of pBC82, which contained the C-terminal region. This plasmid, designated pTEC5, showed only slight EGCase activity when expressed in *E. coli* JM109 cells. Therefore, the fragment including the full-length C9 EGCase gene was prepared by digestion of pTEC5 with *Nco*I and *Eco*RI, its 5′ and 3′ ends were blunted by T4 DNA polymerase treatment, and the resulting fragment was inserted into the *Hinc*II site of pTV118N. The plasmid constructed as C9 EGCase expression vector was designated pTEC6.

Expression and Purification of C9 Recombinant EGCase (rEGCase)—E. coli JM109 cells transformed with pTEC6 were grown at 37°C in 500 ml of Luria-Bertani medium containing 100 μg/ml ampicillin to an optical density (absorbance at 600 nm) of about 0.5. Then isopropylthio-β-D-galactopyranoside (IPTG) was added to the final concentration of 1 mM to cause transcription, and cells were cultured at 37°C for additional 4 h. Cells were harvested by centrifugation, suspended in 18 ml of lysis buffer [10 mM Tris-HCl,

pH 8.0, and 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride], and sonicated for 4 min. Cell debris was removed by centrifugation (6,000 ×*g* for 15 min), and the supernatant was dialyzed for 12 h against 20 mM sodium acetate buffer, pH 5.0. The precipitate was removed by centrifugation (6,000 ×*g* for 15 min), and the supernatant was loaded on a Hi-trap Q column (Amersham Pharmacia Biotech, UK) for ion-exchange chromatography. The enzyme activity was absorbed on the column and eluted by the addition of 0.5 M NaCl. This chromatography increased the specific activities of C9 and M-777 rEGCases by 19- and 20-fold, respectively. These preparations contained no exo- and endoglycosidases acting on GSLs and thus could be used for the determinations of substrate specificity and general properties of rEGCases.

Preparation of Native EGCases—Strains C9 and M-777 were cultured at 25°C for 3 days in liquid medium [1.5% mycological peptone (Oxoid, Great Britain), 0.1% yeast extract (Difco, USA), 0.2% NaCl, and 0.1% bovine brain acetone powder, pH 7.0]. The cultures were centrifuged at 6,000 ×*g* for 20 min, and the supernatants obtained were brought to 75% saturation of ammonium sulfate and allowed to stand overnight. The precipitates were collected by centrifugation, dissolved in 20 mM sodium acetate buffer, pH 5.0, containing 0.1% Triton X-100 and dialyzed against the same buffer. The dialyzed solutions were used as native EGCases.

Enzyme Assay—EGCase activity was measured by two methods.

Assay I (intact GSLs were used as the substrate): The reaction mixture contained 10 nmol of GM1 or other GSLs and an appropriate amount of enzyme in 20 μl of 50 mM sodium acetate buffer, pH 5.0, containing 0.2% Triton X-100. Following incubation at 37°C for the times indicated, the reaction was stopped by heating the mixture in a boiling water bath for 5 min. The reaction mixture was evaporated with a Speed Vac concentrator, and the residue was dissolved in 15 μl of methanol/H_2O (1:1, v/v) and applied to TLC plates, which were then developed with chloroform/methanol/0.02% $CaCl_2$ (4:4:1, v/v). The oligosaccharide released by the action of the enzyme and remaining GSLs were visualized by spraying the TLC plates with orcinol-H_2SO_4 reagent and scanning them with a Shimadzu CS-9300 chromatoscanner with the reflectance mode set at 540 nm. The extent of hydrolysis was calculated as follows: hydrolysis (%) = (peak area of oligosaccharide released) × 100/(peak area of remaining substrate + peak area of oligosaccharide released). One unit of enzyme was defined as the amount capable of catalyzing the hydrolysis of 1 μmol of GM1/min under the conditions described above.

Assay II (NBD-GM1 was used as the substrate): NBD-GM1 was prepared as described elsewhere (*19*). The reaction mixture contained 100 pmol of NBD-GM1 and an appropriate amount of enzyme in 20 μl of 50 mM acetate buffer, pH 5.0, containing 0.2% Triton X-100. Following incubation at 37°C for the times indicated, the reaction was terminated by heating in a boiling water bath for 5 min. The reaction mixture was evaporated with a Speed Vac concentrator, and the residue was dissolved in 15 μl of chloroform/methanol (2:1, v/v) and applied to TLC plates, which were then developed with chloroform/methanol/0.02% $CaCl_2$ (5:4:1, v/v). NBD-Cer released and remaining NBD-GM1 were visualized under UV illumination.

RESULTS

Isolation and Identification of a New EGCase-Producing Actinomycete C9—Among the several microorganisms capable of producing EGCase, strain C9, isolated from land soil, was selected due to its high productivity of the enzyme. C9 was assigned to the genus *Rhodococcus* based on its morphological, physiological, and biochemical characteristics, as shown in "MATERIALS AND METHODS." Phylogenetic analysis based on 16S rDNA showed that C9 was clearly distinguishable from *Rhodococcus* sp. M-777, which was previously isolated as an EGCase-producing actinomycete (Fig. 1).

Molecular Cloning of C9 EGCase Gene—A 500-bp PCR product was obtained from C9 genomic DNA using specific primers containing the NEP sequence. The product was labeled with ^{32}P and used as the probe for Southern blotting. We found that a 4.2-kbp band was hybridized with the probe when the C9 genomic DNA was digested with *Pst*I. A clone, pBC82, containing the 4.2-kbp insert was isolated

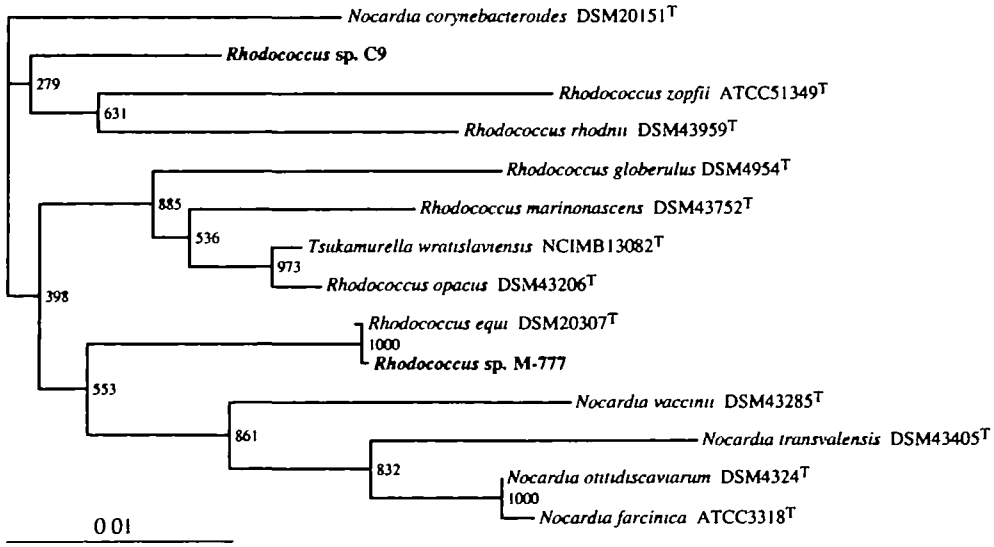

Fig. 1. **Phylogenetic tree based upon 16S rDNA of *Rhodococcus* sp. C9, M-777, and other actinomycetes.** Partial 16S rDNA fragments (about 1 4 kbp) was amplified by PCR using universal primers. Their nucleotide sequences were determined, then subjected to phylogenetic analysis using CLUSTAL W (*16*), with 16S rDNA sequences of other actinomycetes obtained from a DNA database. The phylogenetic tree was constructed by the neighbor-joining method (*26*).

(A)

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                                                              GGGCCCGAACGGATTCCGCCGCGAGTTCGCCGGGTCGACGGACGGCCCGGCCG
CGAGGGTCTCGGTCTCGACGACGGTCGACGCGGGCGGACGCACCCTCGACCTGGTCGTGACGAACGGAGGAACCCGGGATGTGACGGTCGTCGTCGACGGCCGCGGTGGAACGCTGGGTC
CCGGCGCCCGACGCTCGTGGACGGTGCCGTCGACGGACGGCTGGTACCGGTGCGCCGTGACCGTCGACGAGGACACGGACTTCCGGCGCACGCTGGCCGGACACATCGAGAACGGCGAGG
ACAGCGTCAGCCAACCCACCTGACGCGGCACCTGCCACCGTGCGGGCACACGGCCGCACGACCGCCATCTGATCCACACAACCCGTAGGAGGAGCGACAGTGCGTCCAGGAGGAACGACA
GTGCGTCGAACAACAATCGCGTCCCTTGCCGTGGCGGGTCGCTCGTACTCGGGGCCGGTGTGGCCACCGCGCAGAGCAGCTTGCCGGCCACCGGGAGTGACTCGAGCGAGTGGAGCGCA  120
M  R  R  T  R  I  A  S  L  A  V  A  G  S  L  V  L  G  A  G  V  A  T  A  Q  S  S  L  P  A  T  G  S  D  S  S  E  W  S  A    40

TCGGCCTACCTGACGGACGACGCGGGCCGATCCCTGATCCTGCGTGGGTTCAACACGGCATCGAGCGCGAAGAGCACCCCGGACGGCATGCCGATCTTCACCGAGTCCGACCTGGACCGC  240
S  A  Y  L  T  D  D  A  G  R  S  L  I  L  R  G  F  N  T  A  S  S  A  K  S  T  P  D  G  M  P  I  F  T  E  S  D  L  D  R    80

GAGCACGCCGACATGGGAACCAACTTCGTGCGCTTCCTGATCTCCTGGCGTTCGGTGGAACCCGAACCGGGACAGTACGACCAGGCGTATCTGGACCGGGTCGAGCAGCGCGTCGGCTGG  360
E  H  A  D  M  G  T  N  F  V  R  F  L  I  S  W  R  S  V  E  P  E  P  G  Q  Y  D  Q  A  Y  L  D  R  V  E  Q  R  V  G  W   120

TATGCCGAACGCGGCTACAAGGTCATGCTCGACATGCACCAGGACCTCTACTCCGGCGCGATCACCCCCGACGGCAAGACCGGCAACGGCGCGCCGGCATGGGCGACGTACATGGACGGT  480
Y  A  E  R  G  Y  K  V  M  L  D  M  H  Q  D  L  Y  S  G  A  I  T  P  D  G  K  T  G  N  G  A  P  A  W  A  T  Y  M  D  G   160

CTCCCCGTCAACGAGCGGGACAGCTGGGAGCTGTACTACATCGAGCCCGGCGTGATCCGCGCGTTCGACAACTTCTGGAACACCACCGGAAAGCACCCCGAACTCGTCGACCACTACGTG  600
L  P  V  N  E  R  D  S  W  E  L  Y  Y  I  E  P  G  V  I  R  A  F  D  N  F  W  N  T  T  G  K  H  P  E  L  V  D  H  Y  V   200

AATGCCTGGAAGGCCGTCGCGGACCGGTTCGCCGACAACGAGACTGTCGTCGCCTACGACCTGATGAACGAGCCGTGGGGCGGATCCTTGCAGGGACCGGCGTTCGAGGCAGGACCACTC  720
N  A  W  K  A  V  A  D  R  F  A  D  N  E  T  V  V  A  Y  D  L  M [N  E  P] W  G  G  S  L  Q  G  P  A  F  E  A  G  P  L   240

ACCTCGATGTACCAGCGGACCACCGACGCCATCCGACAGGTCGACCAGGACAGCTGGGTCTGCGTCGCCCCGCAGGCTGTCGGCGTCAACCAGGGCATTCCGAGCGCACTCGGCACGATC  840
T  S  M  Y  Q  R  T  T  D  A  I  R  Q  V  D  Q  D  S  W  V  C  V  A  P  Q  A  V  G  V  N  Q  G  I  P  S  A  L  G  T  I   280

GCCGATCCCCGCCAGGGCGCTCGGCGCATCGCCTACTGCCCGCACCTGTATCCCCTCCCCCTCGACCTCGGTGACGGGTACTCGGGGTTCTCGAAGACCCTCACCGACGCCACCATCGAA  960
A  D  P  R  Q  G  A  R  R  I  A  Y  C  P  H  L  Y  P  L  P  L  D  L  G  D  G  Y  S  G  F  S  K  T  L  T  D  A  T  I  E   320

ACCTGGCGCACGAGCATCGAACACGTCGCCGACACCGTTCTCGAGGGTGCACCGGTGATCCTCGGAGAGTTCGGGCTCGACACCACCCTGCCCGGCGCCCAGGACTACCTCGATCGCGTC 1080
T  W  R  T  S  I  E  H  V  A  D  T  V  L  E  G  A  P  V  I  L  G  E  F  G  L  D  T  T  L  P  G  A  Q  D  Y  L  D  R  V   360

TACACCGTCGCTCGCGACATGGGTGCGGGTGTCTCGTACTGGTCGAGCGATCGCGGTCCCTGGGGTCCCTACCTGGAGGACGGGACGCAGACCATCCTCGTCGACACCGTGAACAAGCCG 1200
Y  T  V  A  R  D  M  G  A  G  V  S  Y  W  S  S  D  R  G  P  W  G  P  Y  L  E  D  G  T  Q  T  I  L  V  D  T  V  N  K  P   400

TATCCGCGGGCCGTGGCGGGCATGCCCGTCCGGTGGTCGTCGACCTCCGATCGACTGGACCTGACGTACCGCAACGATCCCGCGGTGACCGCGCCCACCGAGATCTACCTTCCGGCAGCA 1320
Y  P  R  A  V  A  G  M  P  V  R  W  S  S  T  S  D  R  L  D  L  T  Y  R  N  D  P  A  V  T  A  P  T  E  I  Y  L  P  A  A   440

GGATTCCCCGGCGACATCGCCGTCCAGGGGGCGGACGTGGTCGGATGGGACTCACAGAGTCGGCTCCTGACCGTTCGGTCCGCGCCCGACGCGGGTGAGGTGACCGTGACGGTGACGCCC 1440
G  F  P  G  D  I  A  V  Q  G  A  D  V  V  G  W  D  S  Q  S  R  L  L  T  V  R  S  A  P  D  A  G  E  V  T  V  T  V  T  P   480

GCGGCGTGACCCCGTACCTGCGGCCGGCCGGTCAGGCCGGCCGCGGGTGGTGTCACATGTCGAGGCCGAGGTCCAGCACCGTCACCGAATGGGTGAGAGCGCCGACGGCGAGGTAGTCGA 1560
A  A  *                                                                                                                  482

CACCGGTCGCCGCGTAGTCGGCCGCGACGCCCAGGGTCA                                                                                 1599
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(B)

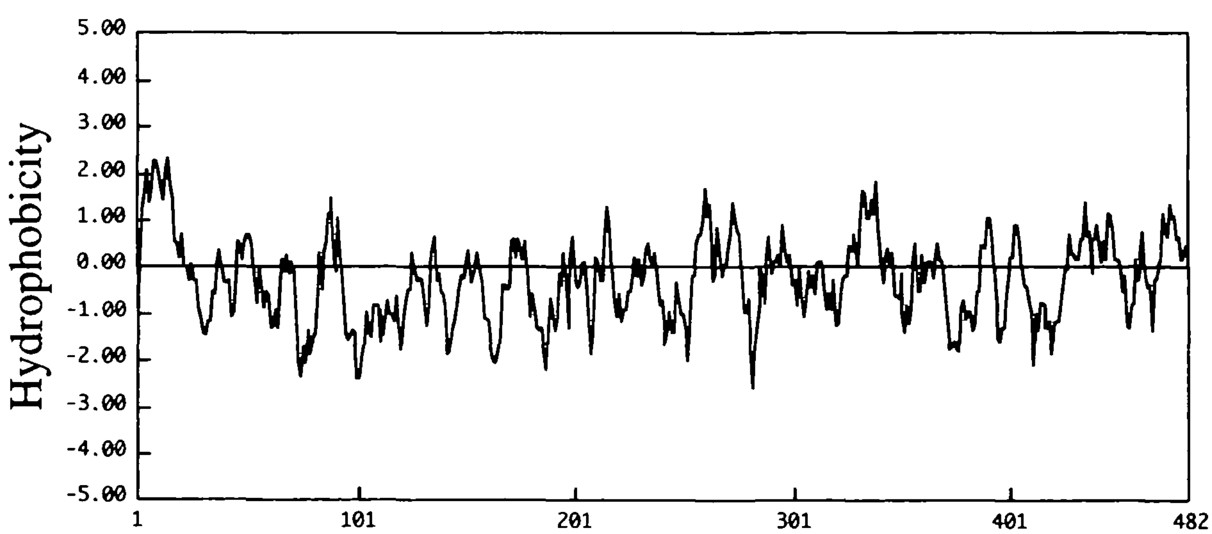

Fig. 2. **DNA and deduced amino acid sequences of a novel EGCase of *Rhodococcus* sp. C9.** (A) The deduced amino acid sequence is shown by one-letter symbols below the nucleotide sequence. The probe used for Southern blotting is single underlined, and the possible Shine-Dalgarno sequences was double underlined. The NEP sequence is boxed. The translation termination codon is indicated by an asterisk (*) (B) Hydropathy analysis of the coding region based upon the deduced amino acids according to Kyte and Doolittle (27).

from the gene library enriched with C9 EGCase gene.

DNA and Amino Acid Sequence Analyses—We sequenced 2,012 nucleotides of pBC82 and found that the open reading frame of the C9 EGCase gene was 1,446 bp long encoding 482 amino acids (Fig. 2, panel A). The NEP sequence, which is the putative active site of EGCase (*10*), is shown by a box on the sequence of C9 EGCase. A hydrophobic region, which is a putative signal sequence (*20*), was found at the 5′ end of the deduced amino acid sequence of C9 EGCase (Fig. 2, panel B). This was consistent with the secretion of C9 EGCase into culture medium. A possible Shine-Dalgarno ribosome binding sequence started 7 bases upstream from the initiation codon GTG. As shown in Fig. 3, the C9 EGCase gene showed 78% and 76% identity to EGCase II of *Rhodococcus* sp. M-777 (M-777 EGCase II) at the nucleotide and amino acid levels, respectively.

Expression of C9 EGCase—The expression vector pTEC6 was constructed by insertion of a fragment of the coding sequence at nucleotide position 94-1446 into the *Hin*cII site of plasmid pTV118N. *E. coli* JM109 cells transformed with pTEC6 were cultured in a medium containing 1 mM isopropylthio-β-D-galactopyranoside. As shown in Fig. 4, C9 rEGCase hydrolyzed NBD-GM1 to produce NBD-Cer (lane 4), whereas the extract from the mock transfectant showed no EGCase activity (lane 3). Recombinant *E. coli* cells produced 14 units of EGCase activity per liter of culture medium when asialo-GM1 was used as the substrate. The molecular mass of C9 rEGCase was estimated to be 58 kDa on SDS-PAGE gels stained with polyclonal antibody against M-777 EGCase II.

Substrate Specificity and Kinetics of C9 rEGCase—The substrate specificity of C9 rEGCase was examined using various sphingolipids and compared with that of M-777 rEGCase II (Table I). Both enzymes hydrolyzed various GSLs other than GlcCer or GalCer, in which the monosaccharide is linked to Cer *via* a β-linkage. Neither enzyme hydrolyzed sphingomyelin or Cer. Gb4Cer was somewhat resistant to hydrolysis by both enzymes, which could thus be classified as type II EGCases. Both enzymes hydrolyzed a-series gangliosides, asialo-GM1, and neolacto-series GSLs to the same extent, but clearly differed in the extent of hydrolysis of b-series gangliotetraosylceramides. GQ1b, GT1b, and GD1b were preferential substrates for M-777 rEGCase II but were strongly resistant to hydrolysis by C9 rEGCase (Table I). This tendency was confirmed by using not only

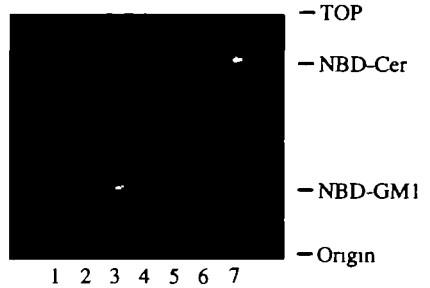

Fig 4. **Hydrolysis of NBD-GM1 by C9 rEGCase.** The expression vector of C9 EGCase was constructed and used for transformation of *E coli* JM109 cells. A 10-μl portion of C9 rEGCase (0.3 mU) was incubated with 100 pmol of NBD-GM1 as a substrate in 20 μl of 50 mM sodium acetate buffer, pH 5 0, containing 0 2% Triton X-100 at 37°C for 3 h The hydrolysis of NBD-GM1 was examined as described in "MATERIALS AND METHODS." Lane 1, mock; lane 2, C9 rEGCase; lane 3, mock + NBD-GM1; lane 4, C9 rEGCase + NBD-GM1, lane 5, M-777 rEGCase II + NBD-GM1; lane 6, NBD-GM1; lane 7, NBD-Cer

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C9     MRRTRIASLAVAGSLVLGAGVATAQSSLPATGSDSSEWSAS--AYLTDDAGRSLILRGFN  58
M-777  MRRTRLVSLIVTGSLVFGGGVAAAQSSLAASGSGSGSGTALTPSYLKDDDGRSLILRGFN  60
       *****  ** *.**** * ***.***** *:** *   .*   :** ** **********

C9     TASSAKSTPDGMPIFTESDLDREHADMGTNFVRFLISWRSVEPEPGQYDQAYLDRVEQRV  118
M-777  TASSAKSAPDGMPQFTEADLAREYADMGTNFVRFLISWRSVEPAPGVYDQQYLDRVEDRV  120
       *******:***** ***.** **.******************** ** *** ******:**

C9     GWYAERGYKVMLDMHQDLYSGAITPDGKTG-------NGAPAWATYMDGLPVNERDSWEL  171
M-777  GWYAERGYKVMLDMHQDVYSGAITPEGNSGNGAGAIGNGAPAWATYMDGLPVEPQPRWEL  180
       ****************:*******:* .*        ***************:    ***

C9     YYIEPGVIRAFDNFWNTTGKHPELVDHYVNAWKAVADRFADNETVVAYDLMNEPWGGSLQ  231
M-777  YYIQPGVMRAFDNFWNTTGKHPELVEHYAKAWRAVADRFADNDAVVAYDLMNEPFGGSLQ  240
       ***:***:*****************:** :**:*********::**********:*****

C9     GPAFEAGPLTSMYQRTTDAIRQVDQDSWVCVAPQAVGVNQGIPSALGTIADPRQGARRIA  291
M-777  GPAFEAGPLAAMYQRTTDAIRQVDQDTWVCVAPQAIGVNQGLPSGLTKIDDPRAGQQRIA  300
       *********::***************:********.*****:** *  * *** * :***

C9     YCPHLYPLPLDLGDGYSGFSKTLTDATIETWRTSIEHVADTVLEGAPVILGEFGLDTTLP  351
M-777  YCPHLYPLPLDIGDGHEGLARTLTDVTIDAWRANTAHTAR-VLGDVPIILGEFGLDTTLP  360
       *********** ***: *:::**** **::**.   * *  **   *:************

C9     GAQDYLDRVYTVARDMGAGVSYWSSDRGPWGPYLEDGTQTILVDTVNKPYPRAVAGMPVR  411
M-777  GARDYIERVYGTAREMGAGVSYWSSDPGPWGPYLPDGTQTLLVDTLNKPYPRAVAGTPTE  420
       **:**..***  **:********** ******* *****:****:********** *

C9     WSSTSDRLDLTYRNDPAVTAPTEIYLPAAGFPGDIAVQGADVVGWDSQSRLLTVRSAPDA  471
M-777  WSSTSDRLQLTIEPDAAITAPTEIYLPEAGFPGDVHVEGADVVGWDRQSRLLTVRTPADS  480
       ********:**    * *:********** ******: *.******** ********:  *:

C9     GEVTVTVTPAA                                                   482
M-777  GNVTVTVTPAA                                                   491
       *.**********
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Fig. 3. **Alignment of C9 and M-777 EGCases.** Identical amino acids are indicated by an asterisk (*), and chemically similar amino acids are by a double dot (.). Gaps inserted into the sequences are indicated by a dash (–). Alignment was performed using CLUSTAL W (*16*).

rEGCases but also native EGCases of these two Rhodococcal strains (data not shown). Interestingly, the degree of sialylation of b-series gangliotetraosylceramides seems to be proportional to that of resistance to the C9 enzyme (Table I). Figure 5 shows the time course for the hydrolysis of GM1, asialo-GM1, and GD1b by both recombinant enzymes, indicating that the C9 enzyme hydrolyzed the neutral GSL much faster than the disialo-GSL, which is a pref-

TABLE I. **Specificity of C9 and M-777 EGCases.**

Substrate	Structure	Hydrolysis(%)	
		C9	M-777
GQ1b	NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1' Cer	4.0	64 2
GT1b	NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1' Cer	14 2	90 0
GD1b	Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1' Cer	23 9	100
GD1a	NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1' Cer	71 4	100
GD3	NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1' Cer	76 9	86 0
GM3	NeuAcα2-3Galβ1-4Glcβ1-1' Cer	92 2	96 1
GM2	GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1' Cer	75 4	73.5
GM1	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1' Cer	76 0	72 6
Asialo-GM1	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1' Cer	96 3	84 4
Gb4Cer	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1' Cer	< 5*	< 5*
nLc$_6$Cer	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1' Cer	61 9	66 9
nLc$_4$Cer	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1' Cer	83 8	92 7
LacCer	Galβ1-4Glcβ1-1' Cer	82 7	100
GlcCer	Glcβ1-1' Cer	0	0
GalCer	Galβ1-1' Cer	0	0
Sphingomyelin	Choline phosphate Cer	0*	0*
Cer	N-stearoyl sphingosine (C18 0, d18 1)	0*	0*

0.3 milliunit of the enzyme was incubated with the appropriate substrate at 37°C for 2 h
*Same amount of enzyme was used but incubated at 37°C for 16 h

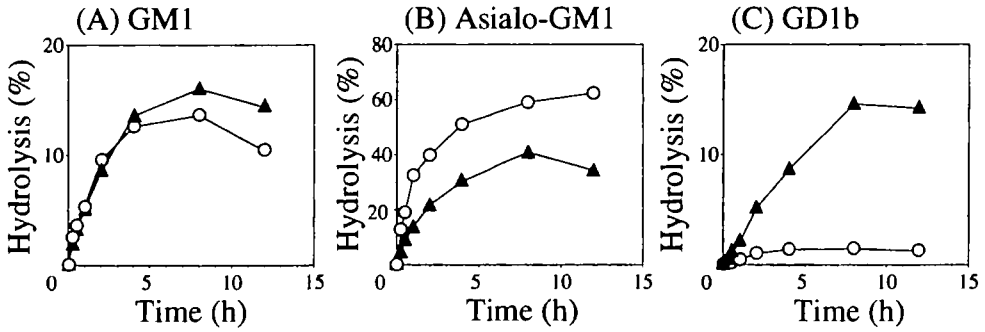

Fig 5. **Time course for hydrolysis of GM1, asialo-GM1, and GD1b by C9 and M-777 rEGCases.** Ten μl of C9 rEGCase or M-777 rEGCase II (10 μU) was incubated with 10 nmol of GM1 (A), asialo-GM1 (B), or GD1b (C) in 20 μl of 50 mM sodium acetate buffer, pH 5 0, containing 0.2% Triton X-100 at 37°C for the times indicated ○, C9 rEGCase, ▲, M-777 rEGCase II.

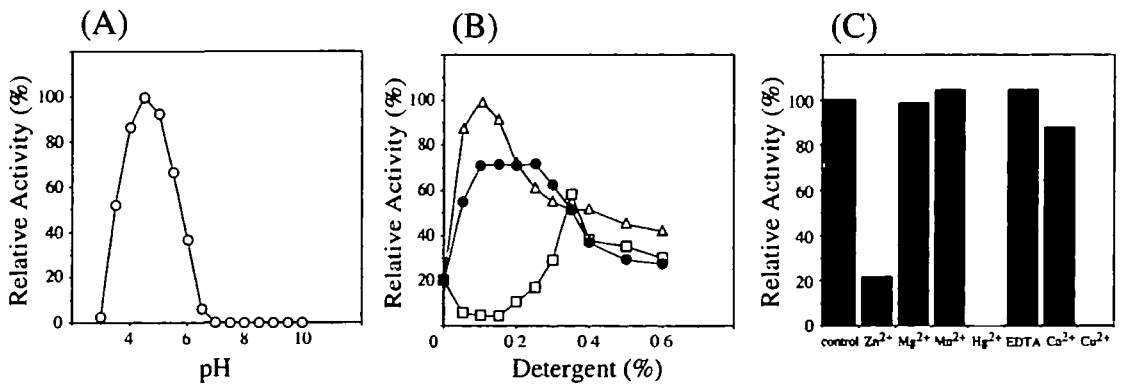

Fig 6. **Effect of various factors on the hydrolysis of asialo-GM1 by C9 rEGCase.** (A) Effects of pH. Ten μl of C9 rEGCase (50 μU) was incubated with 10 nmol of asialo-GM1 in 20 μl of 100 mM of GTA buffer at the indicated pH containing 0 2% Triton X-100 at 37°C for 1 h. (B) Effects of detergents. Ten μl of C9 rEGCase (50 μU) was incubated with 10 nmol of asialo-GM1 in 20 μl of 100 mM of sodium acetate buffer, pH 5.0, containing various concentrations of Triton X-100 (●), Lubrol PX (△), or TDC (□) at 37°C for 1 h. (C) Effects of metal ions. Ten μl of C9 rEGCase (50 μU) was incubated with 10 nmol of asialo-GM1 in 20 μl of 100 mM sodium acetate buffer, pH 5.0, containing various metal ions at 5 mM, and 0.4% Triton X-100 at 37°C for 1 h The enzyme activity was determined as described in "MATERIALS AND METHODS."

erential substrate for the M-777 enzyme. The apparent K_m for asialo-GM1 was 0.50 mM for both enzymed, whereas that for GD1b was 16.5 mM for the C9 enzyme and 0.28 mM for the M-777 enzyme. In conclusion, C9 EGCase showed low affinity for b-series gangliotetraosylceramides compared with the M-777 enzyme.

Properties of C9 rEGCase—The pH optimum for C9 rEGCase was estimated to be pH 4.5 using asialo-GM1 as the substrate (Fig. 6, panel A). C9 rEGCase was activated by the addition of Triton X-100 and Lubrol PX. The optimum concentrations of Triton X-100 and Lubrol PX were 0.1–0.25% and 0.1%, respectively. TDC activated the C9 enzyme at the concentration of 0.35–0.4% but inhibited it at lower concentrations (Fig. 6, panel B). M-777 EGCase II was activated by Triton X-100 and TDC but not by Lubrol PX (*21*). Among metal ions examined, Hg^{2+} and Cu^{2+} completely inhibited the C9 enzyme activity and Zn^{2+} and Ca^{2+} inhibited it by 22% and 87%, respectively. On the other hand, Mg^{2+}, Mn^{2+}, and EDTA did not show significant effects on C9 EGCase activity (Fig. 6, panel C).

DISCUSSION

The glycohydrolase that hydrolyzes the β1,1glycosidic linkage between oligosaccharide and Cer of GSLs is named endoglycoceramidase [EC 3.2.1.123], or EGCase for short. EGCase does not hydrolyze sphingomyelin, phosphorylcholine is linked to Cer, or cerebroside, in which a monosaccharide is linked to Cer by a β-linkage. The smallest sugar unit susceptible to hydrolysis by EGCase is lactose. Three molecular species of EGCase (I, II, III) with different specificities were detected in the culture supernatant of *Rhodococcus* sp. M-777. EGCases I and II were capable of hydrolyzing the GlcCer linkage of ganglio-type, lacto-type, and globo-type GSLs. Globo-type GSLs were strongly resistant to hydrolysis by EGCase II in comparison with EGCase I. It was revealed that the species and anomeric linkage of the sugar moiety neighboring LacCer determine the susceptibility to hydrolysis by the enzymes. The α-linked galactose neighboring LacCer (in globo-type GSLs) may lessen the access of EGCase II, but not EGCase I, to the substrate. EGCase III specifically hydrolyzes the GalCer linkage of gala-type GSLs, which are not hydrolyzable at all by EGCase I or II. The results obtained in this study clearly indicate that C9 EGCase is a type II enzyme. Interestingly though, the substrate specificity of C9 EGCase for b-series gangliotetraosylceramides is completely different from that of M-777 EGCase II, and thus C9 enzyme seems to be a novel EGCase.

We previously reported that part of the amino acid sequence deduced from the EGCase II gene of *Rhodococcus* sp. M-777 was homologous to that of the active site region of family A cellulases (endo-1,4-β-glucanases), and the NEP sequence, which is commonly conserved in endo-1,4-β-glucanases, was also found in the M-777 enzyme (*9*). It was demonstrated that the Glu residue in the NEP sequence was the active site of endo-1,4-β-glucanases (*22–24*) and possibly of EGCase II (*10*). PCR products of 500 bp were specifically generated from templates of genomic DNAs of EGCase-producing microorganisms, not only *Rhodococcus* sp. C9 and M-777 but also several other strains of authentic *Rhodococcus* species, when PCR was performed using a specific primer containing the NEP sequence. Therefore, the NEP sequence seems to be commonly conserved in EGCases.

Activities of EGCase, also called Cer glycanse, are found in not only procaryotes but also eucaryotes. Recently, Basu *et al.* reported the possible presence of EGCase in mammals (*25*). However, the biological significance of eucaryotic EGCase is still unclear. In general, GSLs in vertebrates are recycled between the plasma membrane and intracellular organs, then finally transported to lysosomes, where they are hydrolyzed sequentially from the non-reducing end by exo-type glycosylhydrolases. The presence in vertebrates of a novel metabolic pathway of GSLs involving EGCase and/or the signal transduction pathway mediated by GSL-derived Cer remains to be clarified. PCR using the NEP sequence as a primer could be useful for cloning cDNA encoding EGCase in eucaryotic cells.

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