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**Cloning and sequencing of the cellobiose 2-epimerase gene from an  
obligatory anaerobe, *Ruminococcus albus***

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## Abstract

Cellobiose 2-epimerase (EC 5.1.3.11) was first identified in 1967 as an extracellular enzyme that catalyzes the reversible epimerization between cellobiose and 4-*O*- $\beta$ -D-glucopyranosyl-D-mannose in a culture broth of *Ruminococcus albus* 7 (ATCC 27210<sup>T</sup>). Here, for the first time, we describe the purification of cellobiose 2-epimerase from *R. albus* NE1. The enzyme was found to 2-epimerize the reducing terminal glucose moieties of cellotriose and cellotetraose in addition to cellobiose. The gene encoding cellobiose 2-epimerase comprises 1170 bp (389 amino acids) and is present as a single copy in the genome. The deduced amino acid sequence of the mature enzyme contains the possible catalytic residues Arg52, His243, Glu246 and His374. Sequence analysis shows the gene shares a very low level of homology with *N*-acetyl-D-glucosamine 2-epimerases (EC 5.1.3.8), but no significant homology to any other epimerases reported to date.

*Keywords:* cellobiose 2-epimerase; *Ruminococcus albus*; rumen; cloning; sequencing

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The first stomach of ruminants, known as the rumen, contains a large population of microbes, including bacteria, protozoa and fungi. Most of these microbes are considered to be cellulolytic and obligate anaerobes. Several cellulolytic enzymes have been studied whilst attempting to analyze the anaerobic ecosystem of the rumen [1–4]. Since Bryant and Burkey [5] established effective methods to isolate and cultivate ruminal microbiota, a number of cellulolytic rumen bacteria, such as the genera *Ruminococcus*,

*Clostridium*, *Lactobacillus* and *Prevotella*, have been isolated [6–8]. In addition to *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*, *Ruminococcus albus* is one of the main cellulolytic bacteria in the rumen. Indeed, *R. albus* produces various extracellular cellulolytic enzymes [2, 3, 4, 9, 10]. Furthermore, the mechanism of adhesion of cellulose to the cell surface of *R. albus* has been extensively studied [11–15]. The reported cellulolytic enzymes of *R. albus* include an endo- $\beta$ -1,4-glucanase (EC 3.2.1.4), an exo-type cellobiosidase (EC 3.2.1.91) and a  $\beta$ -glucosidase (EC 3.2.1.21) [16–18].

In 1967, Tyler and Leatherwood [19] described an enzyme activity from the culture broth of *R. albus* 7 (*R. albus* ATCC27210<sup>T</sup>) that mediates the epimerization of cellobiose and named it cellobiose epimerase (CE). This enzyme 2-epimerizes cellobiose and generates 4-*O*- $\beta$ -D-glucopyranosyl-D-mannose (Glc-Man) in a reversible manner. However, in the last four decades, there have been no reports describing the purification, enzymatic properties or gene sequence of cellobiose 2-epimerase (EC 5.1.3.11). Here, we describe the purification to homogeneity of a CE (CE-NE1) from *R. albus* NE1 and the cloning and sequencing of the corresponding gene (*ce-ne1*). We also show that CE-NE1 is an intracellular, rather than extracellular, enzyme.

## **Materials and methods**

*Bacterial strains and propagation.* *R. albus* NE1, originally isolated by H. Taguchi from cow rumen fluid in this laboratory, was deposited as a patented strain (FERM P-21036) in the National Institute Bioscience and Human Technology of the Agency of Industrial Science and Technology of Japan. It was propagated anaerobically in 20 L of the medium of Tyler and

Leatherwood [19] at 37°C for 48 h. *Escherichia coli* JM109 for plasmid preparation and sequencing was grown aerobically at 37°C for 16 h in Luria-Bertani broth. As required, the broth was solidified by inclusion of 1.5% agar. When necessary, the medium was supplemented with ampicillin (100 µg/mL) for selection of clones.

*Purification of CE-NE1.* Cells were suspended in 20 mM MES buffer (pH 6.0) plus 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethanesulfonyl fluoride. The suspension was passed through a French press (Ohtake Works, Tokyo, Japan) and then cell debris subsequently removed by centrifugation (6000 × g, 10 min, 4°C). The supernatant (210 mL) was then loaded directly onto coupled columns (15 mm × 150 mm each) of CM Sepharose Fast Flow and DEAE Sepharose CL-6B (GE Healthcare Bio-Sciences, Piscataway, NJ). Proteins were eluted with a linear gradient of NaCl (0–0.5 M), and the active fractions were pooled and combined (100 mL). Ammonium sulfate was then added to 45% saturation, and the solution was applied to a column (15 mm × 150 mm) of Toyopearl Ether-650M (Tosoh, Tokyo, Japan) that had been equilibrated with 20 mM MES buffer (pH 6.0) plus 1 mM EDTA, 1 mM dithiothreitol and 45% ammonium sulfate. Proteins were eluted with a linear gradient of ammonium sulfate (from 45% to 0%), and the active fractions (26 mL) were pooled and combined. This solution was dialyzed against 5 mM phosphate buffer (pH 6.0) plus 1 mM EDTA and 1 mM dithiothreitol. The retentate was applied directly to a column (15 mm × 150 mm) of hydroxyapatite (Seikagaku Kogyo, Tokyo, Japan) equilibrated with the same buffer, and eluted with a linear gradient of phosphate buffer (pH 6.0) from 5 mM to 200 mM. The active fractions (70 mL) were pooled and applied directly to a column (6.4 mm × 30 mm) of Resource Q (GE Healthcare Bio-Sciences) equilibrated with 20 mM MES buffer (pH 6.0) plus 1 mM EDTA and 1 mM dithiothreitol. Proteins were eluted with a linear gradient of NaCl (0–0.4 M) in the buffer at a flow rate of 1.5 mL/min in a fast protein chromatography system (GE Healthcare Bio-Sciences), and the active fractions (2.2

mL) were pooled and combined. Finally, the solution was fractionated on a Superdex 200 HR 10/30 (GE Healthcare Bio-Sciences) column (10 mm × 300 mm) equilibrated with MES buffer (pH 6.0). Active fractions were pooled and concentrated by ultrafiltration (Microcon YM-30, Millipore, Billerica, MA). The concentrate was used as the final enzyme preparation.

*Enzyme assay and protein determination.* The initial rate of CE activity was assayed by the following method. The reaction mixture containing 250  $\mu$ L of 200 mM glycylglycine (pH 7.8), 100  $\mu$ L of 25 mg/mL cellobiose and 150  $\mu$ L of the suitably diluted enzyme solution was incubated at 30°C for a defined length of time. The reaction was stopped by boiling for 5 min, and the reaction mixture was diluted 5-fold with distilled water. The diluted reaction mixture (500  $\mu$ L) was passed through a column (4 mm × 10 mm) of AG501-X8 resin (Bio-Rad, Hercules, CA), and 90  $\mu$ L aliquots of the eluate were mixed with 10  $\mu$ L of the internal standard maltitol (2 mg/mL). The production of Glc-Man from cellobiose was measured by using a Sugar SP0810 HPLC column (8.0 mm × 300 mm; Shodex, Tokyo, Japan) with distilled water as the mobile phase (0.8 mL/min) and an evaporative light scattering detector (ELD2000ES; Alltech Associates, Deerfield, IL) at 115°C and an air flow rate of 3.2 L/min. The standard curve was drawn by using authentic Glc-Man. One unit of enzyme activity was defined as the amount of protein that formed 1  $\mu$ mol of Glc-Man per min. The protein concentration was determined according to the method of Bradford [20] with bovine serum albumin as the standard.

*DNA extraction and preparation of probe.* All primers used in this study are shown in Table 1. The chromosomal DNA of strain NE1 was isolated by the cetyltrimethylammonium bromide procedure [21]. Preparation of plasmid DNA, restriction digestion, ligation and transformation of *E. coli* were performed essentially by the method of Sambrook et al. [22]. Analysis of genomic information of *R. albus* 8 from TIGR (<http://www.tigr.org/>) using the N-terminal sequence obtained from purified CE-NE1 allowed us to identify a hypothetical protein

named Nelp8, similar to an *N*-acetyl-D-glucosamine 2-epimerase (AGE; EC 5.1.3.8). PCR primers were designed to amplify DNA encoding the Nelp8-like sequence from the NE1 genome. The PCR product was cloned by continuous reactions with two primer sets (NAc2epi S and NAc2epi AS for first PCR; NAc2epi S and NAc2epi AS nes for nested PCR) into a pBluescript II SK(+) vector (Stratagene Cloning Systems; La Jolla, CA) and then sequenced.

*Southern blotting.* The probe for Southern blot analysis was prepared from the cloned PCR fragment and primers (epi sen1 and epi ant1) with a digoxigenin DNA-labeling kit (Roche, Basel, Switzerland). The genomic DNA was digested with *Bgl*II, *Eco*RI, *Eco*RV, *Pst*I or *Pvu*II. Ten micrograms of the digested genomic DNA was separated by 0.7% agarose gel electrophoresis and blotted onto a Hybond-N<sup>+</sup> membrane (GE Healthcare Bio-Sciences). The membrane was fixed under UV irradiation. Hybridization was performed in 5×SSC, 0.1% lauroylsarcosine, 0.02% SDS, and 1% blocking agent (Roche) with the digoxigenin-labeled probe. The hybridized membrane was washed in 0.6×SSC and 0.02% SDS at 50°C. Non-R1 chemiluminescent detection with an alkaline phosphatase-conjugated anti-digoxigenin antibody and a CDP-Star chemiluminescent substrate was performed according to the manufacturer's instructions (Roche).

*Cloning and sequencing of CE-NE1 gene.* The genomic DNA was digested with *Eco*RI, and then incubated for 16 h at 16°C in a Ligation Mix (TaKaRa Bio, Kyoto, Japan). Using this ligation solution as a template, inverse PCR was carried out with two primer sets (epi inverse S and epi inverse AS for first PCR; epi inverse S nes and epi inverse AS nes for nested PCR) to amplify the region that contained the *ce-ne1* gene. The amplified products were then cloned into pBluescript II SK(+) vectors and introduced into *E. coli* JM109 cells. The amplified products were directly used as template to sequence the *ce-ne1* gene using primers epi inverse S, epi inverse AS, epi sen1, epi sen2, and epi ant1 (Table 1).

Nucleotide (nt) sequences were analyzed by the dideoxynucleotide chain termination method using an automatic DNA sequencer (ABI PRISM 310; Applied Biosystems, Foster City, CA). Nucleotide and amino acid (aa) sequence databases were searched using the BLASTN and BLASTX programs (NCBI and DDBJ; <http://www.ncbi.nlm.nih.gov/BLAST/> and <http://blast.ddbj.nig.ac.jp/>, respectively).

*Structure-based alignment.* The aa sequence alignment of CE-NE1 with porcine kidney AGE (PDB code 1fp3) was done manually. The secondary structures of AGE were maintained where the insertion and deletion occurred in both sequences. The secondary structures of CE-NE1 were assigned by the Kabsch-Sander method [23].

*Thin-layer chromatography of epimerized products.* Thin-layer chromatography (TLC) was used to identify the products generated from cellobiose, cellotriose, cellotetraose and *N*-acetyl-D-glucosamine (Seikagaku Kogyo) by CE-NE1. Reactions were performed at 25°C for an appropriate length of time in a 25  $\mu$ L reaction mixture containing 15  $\mu$ L of 100 mM Tris-maleate (pH 7.0), 5  $\mu$ L of a 100 mM cello-oligosaccharide and 5  $\mu$ L of enzyme solution. Reactions were stopped by boiling for 5 min. Aliquots (3  $\mu$ L) of the reaction mixture were spotted on TLC plates (Silica gel 60F254; Merck, Whitehouse Station, NJ), and developed in a solvent system of 2-propanol/1-butanol/H<sub>2</sub>O (7:2:1, v/v). The chromatograms were developed by color reaction using the anisaldehyde-sulfuric acid procedure. When necessary, 50  $\mu$ L of 8 M trifluoroacetic acid was added to 50  $\mu$ L (262  $\mu$ g) of the concentrate and kept at 100°C for 3 h to completely hydrolyze the Glc-Man. After the solvent was replaced with distilled water in a centrifugal concentrator, the hydrolyzate was spotted on a TLC plate. Total carbohydrate was determined by the phenol-sulfuric acid procedure with glucose as the reference standard.

*SDS-polyacrylamide gel electrophoresis.* SDS-polyacrylamide gel electrophoresis was performed using 10% polyacrylamide slab gels. Protein molecular mass standards (14.4–97.4



kDa) were obtained from Bio-Rad.

*Analysis of N-terminal aa sequence.* To determine the N-terminal aa sequence, protein bands from SDS gels were transferred to a PVDF membrane with a Trans-blot Semi-dry Transfer Cell (Bio-Rad). The aa sequence was analyzed using an automatic protein sequencer (Procise 491; Applied Biosystems).

*<sup>13</sup>C-NMR spectrometric analysis of epimerization product of cellotriose.* The reaction was carried out overnight at 25°C in a mixture containing 32 mL of 100 mM Tris-maleate (pH 7.0.), 6.2 mL of 100 mM cellotriose, and 1.6 mL of enzyme (10.9 µg). The reaction was stopped by boiling the mixture for 5 min. The reaction mixture was spotted on a TLC plate (Silica gel 60F254), and the narrow guide strip was colored for the measurement of carbohydrate mobility. Then, the reaction product was scraped from the TLC plate. The product was extracted in distilled water twice, and the extract was concentrated by rotary evaporation. The cellotriose product (5 mg) was analyzed by <sup>13</sup>C-NMR spectrometry (BRUKER AMX500, Bruker, Germany), using 2, 2, 3, 3-D4 sodium 3-3-(trimethylsilyl)propionate as the external standard.

*Accession numbers.* The complete nucleotide sequences of CEs of *R. albus* NE1 and *R. albus* 7 (ATCC 27210<sup>T</sup>) have been submitted to the DDBJ/EMBL/GenBank DNA databases under accession nos. **AB301953** and **AB301954**, respectively.

## Results and discussion

### *Purification of CE-NE1*

CE-NE1 was purified to homogeneity from a cell-free extract of *R. albus* NE1. The overall yield was only 0.005% of the initial total protein (1.7 g). Using our purification procedure, the specific activity toward cellobiose was 3–5 units/mg at a pH optimum 7.8 in 100 mM glycylglycine buffer. The molecular mass of the purified protein was approximately 43.1 kDa by SDS-PAGE (Fig. 1A). *N*-terminal sequencing yielded 17 unambiguous aa residues (Met-Met-Ile-Ser-Glu-Ile-Arg-Asn-Glu-Leu-Thr-Glu-His-Ile-Ile-Pro-Phe). A database search of the genome sequence of *R. albus* 8 (<http://www.tigr.org/>) using the *N*-terminal sequence as a query identified a hypothetical protein named Nelp8. Twelve out of the 17 *N*-terminal aa residues of Nelp8 were found to be identical (**Met-Met-Lys-Glu-Glu-Val-Lys-Asn-Glu-Leu-Thr-Ser-His-Ile-Ile-Pro-Phe**; bold letters indicate aa residues identical to those of CE-NE1). AGE is known to yield *N*-acetyl-D-mannosamine by inverting the C-2 of *N*-acetyl-D-glucosamine [24].

### *Substrate specificity of CE-NE1*

Unidentified CE products were detected by TLC when cellobiose, cellotriose or cellotetraose were used as substrates, as shown in Fig. 1B. The product obtained from cellobiose was identified as Glc-Man, as judged by comparing its mobility with authentic Glc-Man on TLC. Furthermore, the chemically hydrolyzed products were glucose and mannose (data not shown). To confirm whether the reducing terminal

glucose is precisely 2-emimerized or not, the product from cellotriose was analyzed by  $^{13}\text{C}$ -NMR spectroscopy. The spectrum of the product completely coincided with that of 4-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-mannose (Glc-Glc-Man) [25]. From these results, the product of cellotetraose is undoubtedly 4-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-mannose (Glc-Glc-Glc-Man). The yield of purified CE obtained in this study was too low to further examine other enzymatic and physicochemical properties.

#### *Nucleotide and deduced amino acid sequences of CE-NE1 gene*

A multiple-alignment of the aa sequence of Nelp8 with other AGEs was performed. Some conserved regions were identified, and primers were designed to these conserved sequences (NAc2epi S, NAc2epi AS, NAc2epi AS nes) in order to amplify *ce-ne1* using *R. albus* NE1 genomic DNA as template. Using NAc2epi S and NAc2epi AS primers, an 800-bp fragment was obtained. Nested PCR was then performed with NAc2epi S and NAc2epi AS nes primers, which yielded a 700-bp fragment. The PCR product showed 70% identity to the corresponding region of Nelp8. Based on this partial sequence, we designed primers (epi sen1 and epi ant1) to prepare a suitable hybridization probe for Southern blot analysis of *R. albus* NE1 genomic DNA. A single cross-hybridizing *Eco*RI band of approximately 4-kbp was detected, suggesting that the gene is present as a single copy in the *R. albus* NE1 genome (Fig. 2).

The 4-kbp band was subsequently sequenced. Nucleotide analysis identified an open reading frame (ORF, 1170 nt) starting from a putative ATG start codon to a TGA stop codon (**AB301953**). The deduced aa sequence of the ORF contained 389 aa residues

with a calculated molecular mass and isoelectric point of 45217.4 Da and pH 4.69, respectively. The calculated molecular mass was close to the 43.1 kDa of the native CE-NE1 purified from *R. albus* NE1 cells. The *N*-terminal sequence Met-Met-Ile-Ser-Glu-Ile-Arg-Asn-Glu-Leu-Thr-Glu-His-Ile-Ile-Pro-Phe completely coincided with that of the purified enzyme. The entire aa sequence of Nelp8 in the genome of *R. albus* 8 showed 82% identity to that of CE-NE1. In a 50-fold concentrated crude extract of *R. albus* NE1, CE activity was detected on TLC after 6 h incubation. However, under identical conditions no AGE activity could be detected, suggesting that Nelp8 may encode CE rather than AGE. Furthermore, the deduced aa sequence of CE-NE1 showed very low homology to AGE from porcine kidney (**D83766**) [24, 26] and *Anabaena* sp. CH1 (**DO661858**) [27] with only 15% and 17% identity, respectively. Thus, we conclude that the cloned sequence encodes CE. The CE gene from *R. albus* 7 (ATCC 27210<sup>T</sup>) was also cloned and sequenced (**AB301954**) and showed 100% identity to that of CE-NE1.

Because CE-NE1 was extracted and purified from a cell extract, rather than the culture broth, the enzyme must be intracellular. Furthermore, the aa sequence does not contain any signal sequence for secretion. Thus, the CE of *R. albus* must be distinct from the extracellular activity reported by Tyler and Leatherwood [19].

Although AGEs from *Clostridium phytofermentans* ISDg (**Q1FKV5**) and *Caldicellulosiruptor saccharolyticus* DSM 8903 (**Q2ZKZ6**) showed 50% and 44% identity to CE-NE1, respectively, these are hypothetical proteins, and their catalytic properties have not yet been identified. CE-NE1 exhibited no significant homology to the reported epimerases such as ribulose-phosphate 3-epimerase from *Bacillus subtilis* (**Q34557**), UDP-glucose 4-epimerase from *Bacillus halodurans* (**Q9KDV3**), aldose 1-epimerase from *E. coli* (**P0A9C3**), UDP-*N*-acetylglucosamine 4-epimerase from *E. coli*

O157 (**Q8X7P7**), *N*-acylglucosamine-6-phosphate 2-epimerase from *Salmonella paratyphi* (**Q5PG85**), UDP-glucuronate 5-epimerase from *Rhizobium meliloti* (**O54067**), and L-ribulose-5-phosphate 3-epimerase from *E. coli* (**P37679**). This negative result suggests that the gene for CE has evolved from a different ancestral gene to those for the reported epimerases.

#### *Structure-based alignment of CE-NE1 and porcine AGE*

The nt sequence and 3D structure of an AGE from porcine kidney has been determined [24, 26]. We performed a structure-based alignment of the porcine AGE and CE-NE1 (Fig. 3). From the secondary structure assignment [23], CE has an  $(\alpha/\alpha)_6$  core structure, similar to the AGE. Our analysis revealed several candidate catalytic residues Arg52, His243, Glu246 and His374 of CE-NE1, corresponding to Arg60, His248, Glu251 and His382 of the porcine kidney enzyme, which are involved in the catalysis of the AGE. The charged aa residues on the active site cleft in the AGE are also partly conserved in CE-NE1. According to Lee et al. [27], the two His residues play a key role in reversible conversion in the case of an AGE from *Anabaena* sp. CH1, as revealed by site-directed mutagenesis experiments. Intriguingly, although the AGEs from porcine kidney and *Anabaena* sp. CH1 display <20% identity to CE-NE1, all these enzymes specifically discriminate 2-epimerization at the C-2 positions of the corresponding substrates.

*R. albus* is a potent cellulolytic enzyme producer. However, the physiological role(s) of an intracellular CE has not yet been clarified, although CE-NE1 is induced by cellulose and cellobiose (unpublished results). Indeed, the metabolic fates of the 2-

epimerized cello-oligosaccharide derivatives are quite interesting and puzzling with respect to cellulose catabolism by *R. albus*. In order to clarify these issues we are now attempting to create various mutant strains and mutant enzymes and to determine the 3D structure of recombinant CE-NE1.

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## Figure legends

**Fig. 1.** SDS-PAGE of purified CE-NE1 and TLC analysis of CE products. A. The purified enzyme (0.1  $\mu\text{g}$ ) was electrophoresed using a 10% acrylamide slab gel and visualized by the method of Bradford [20]. The molecular mass of purified CE-NE1 is indicated by the arrow. Lane 1, molecular size markers; lane 2, purified CE. B. The substrates used were cellobiose (1), cellotriose (2), and cellotetraose (3). After overnight reaction at 25°C, 2.5  $\mu\text{l}$  aliquots were spotted on a TLC plate. The spot on the left in each lane is that of negative control (without enzyme).

**Fig. 2.** Southern blot analysis. Genomic DNA prepared from *R. albus* NE1 (3  $\mu\text{g}$ ) was digested with *EcoRI* and fractionated on a 0.7% agarose gel (lane 1). DNA fragments were transferred to a nylon membrane and processed for Southern blotting using the DIG-labeled probe (lane 2). M, molecular size markers.

**Fig. 3.** Structure-based alignment of amino acid sequences of CE-NE1 and porcine kidney AGE. An alignment of the aa sequences is shown with  $\alpha$ -helices (single underlines) and  $\beta$ -sheets (double underlines) beneath the sequences. Asterisks under the sequences indicate conserved aa residues. In the case of porcine AGE, the aa residues designated by filled circles are possible catalytic residues, and those shown in boldface are charged aa in the active site cleft [26].

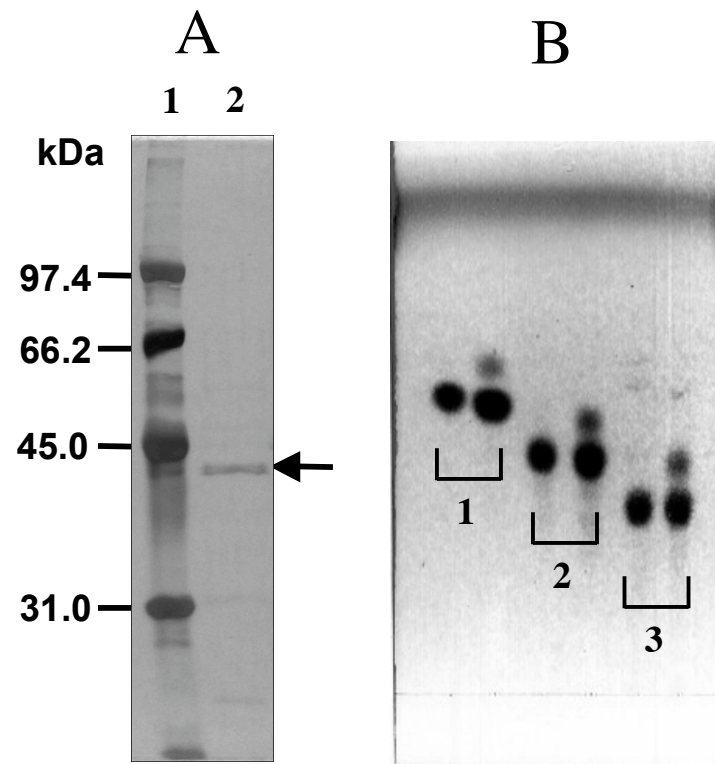


Fig. 1

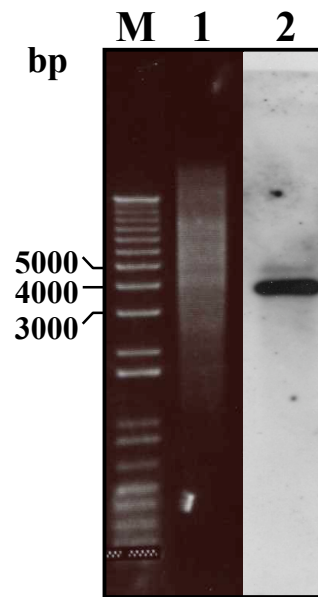


Fig. 2

CE-NE1	<u>MMISEIRQELTDHIIPFWNKLRDDENGGFYGYLSYGLELDKKADKGVILHSRILWFYSNAYMTLG</u>	65
AGE	<u>MEKERETLQAWKERVGOELDRVMAFWLEHSHDREHGGFFTCCLRDRV-YDDLKYVWLOGROVWMYCRLYRKLE</u>	73
	*** * * * * * * * * * * * * *	
CE-NE1	<u>---GDELLDNAKHAYEFIKNNCIDYEY-GGVYWMDFEGKPADTMKHTYNI AF AIYALSSYYRASGDKEALALA</u>	135
AGE	<u>RFHRPELLDAAKAGGEFLLRHARVAPPEKKCAFVLT RDGRPVKVORSIFSECFYTMAMNELWRVTAEARYQSEA</u>	147
	**** * * * * * * * * * * * *	
CE-NE1	<u>YRLFEDIEKNTLYEYGYREAFDRQWRLVDNEALSENGLKADKTMNAILHLIEAYTELYKADGNEKVADRLKFOL</u>	209
AGE	<u>VDMMDQIVHWVREDPSGLGRPOL-----PGAVASESM AVPMMLLCLVEQLGEEDEELAGRYAQLGHCARRILO</u>	216
	* ** * * *	
CE-NE1	<u>GOMRDIVYTPDTNALKVFFDTAFNLVGD IHSYGHDI EATWLM DRACDVLGDEDLKKQFAEMDLKISHNIQDIAL</u>	283
AGE	<u>HVQRD-GOAVLENVSEEDGE-ELSGCLGRHQNPGHALEAGWFLLRHSSRSGDAKLRAHVIDTFLLLPFRSGWDAD</u>	288
	** * * * * * * * * * *	
CE-NE1	<u>EDGALNNERDKNEID-----KTRVWVWQAEAVVGF INAYQHSGDEKFL ESAKSVWENIKEYIIDKREGGEWYS</u>	351
AGE	<u>-HGGLFYFQDADGLCPTQLEWAMKLVVPHSEAMIAFLMGYSESGDPALLRLFYQVAEYTFROFRDP-EYGEWFG</u>	360
	* * * * * * * * * * *	
CE-NE1	<u>EVTFDHTPHDYKETVGPWKCPYHNGRMCMEVITRGVDI</u>	389
AGE	<u>YLNRE-GKVALTIKGGPFKGC FHVPRCLAMCEEMLSALLSRLA</u>	402
	** * * *	

Fig. 3

Table 1

Primers used in this study

Primer	Sequence (5'→3')	Comments
NAc2epi S	GATGATGAGAACGGCGGCTTT	For isolation of a partial fragment of <i>ce-nel</i>
NAc2epi AS	GCTTCCGCCTGCACCCACCATA	For isolation of a partial fragment of <i>ce-nel</i>
NAc2epi AS nes	ATCAGCCATGTGGCTTCAATGTCGTGTCCG	For isolation of a partial fragment of <i>ce-nel</i>
epi sen1	CGCTTATATGACACTGGGCG	For Southern blot probe and sequencing of <i>ce-nel</i>
epi ant1	TCAGCCTGTCAGCTACCTTC	For Southern blot probe and sequencing of <i>ce-nel</i>
epi inverse S	GAAGCGTATACCGAGCTGTACAAGGC	For the first inverse PCR and sequencing of <i>ce-nel</i>
epi inverse AS	CCATCATCCAGTATACTCCACCGTATTC	For the first inverse PCR and sequencing of <i>ce-nel</i>
epi inverse S nes	CGAGAAGGTAGCTGACAGGCTGAAGTTCC	For the nested inverse PCR
epi inverse AS nes	CAGTTGTTCTTTATGAACTCATAAGCGTG	For the nested inverse PCR
epi sen2	GAACGAGATAGACAAGACCCGCGTATG	For sequencing of <i>ce-nel</i>