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Sequence-Based Screening for a Putative γ-Butyrobetaine Hydroxylase Gene from *Neurospora crassa*

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Abstract The last step in L-carnitine biosynthesis in eukaryotic organisms is mediated by γ-butyrobetaine hydroxylase (EC 1.14.11.1), a dioxygenase that converts γ-butyrobetaine to L-carnitine. This enzyme was previously identified from rat liver and humans, and the peptide sequence of human γ-butyrobetaine hydroxylase was used to search the *Neurospora crassa* genome database, which led to an identification of an open reading frame (ORF) consisting of 1,407 bp encoding a polypeptide of 468 amino acids. When this protein was expressed in *Saccharomyces cerevisiae*, the crude cell-free extract exhibited γ-butyrobetaine hydroxylase activity.

Key words: L-Carnitine, γ-butyrobetaine hydroxylase, cDNA library, *Saccharomyces cerevisiae*, *Neurospora crassa*

L-Carnitine (3-hydroxy-4-trimethylammonium-butyrate) is a chiral molecule required for the transport of activated fatty acids across the inner mitochondrial membrane into the matrix for β-oxidation [3]. L-Carnitine can be synthesized from lysine as a precursor by many eukaryotic organisms, and the identity of the intermediate metabolites of the L-carnitine biosynthetic pathway has been established in the filamentous fungus *Neurospora crassa* [5, 11, 21]. It has been demonstrated that L-carnitine is synthesized from lysine by sequential reaction of five enzymes in *N. crassa*; S-adenosylmethionine-6-*N*-L-lysine methyltransferase, ε-*N*-trimethyllysine hydroxylase (EC 1.14.11.8), β-hydroxy-ε-*N*-trimethyllysine aldolase, γ-trimethylaminobutyraldehyde dehydrogenase (EC 1.2.1.47), and γ-butyrobetaine hydroxylase (EC 1.14.11.1) [5]. More recently, enzymes required for the catalysis of the reactions in L-carnitine biosynthesis have been characterized at the molecular level in different kinds of prokaryotic [6, 14] and eukaryotic organisms [20, 23, 24]. However, most of the enzymes responsible for the L-carnitine biosynthesis in *N. crassa* have not yet been characterized at the molecular level.

L-Carnitine has a wide range of applications in pharmaceuticals, food products, and feed additives, since it first became commercially available in the 1980s. However, the production of L-carnitine has been mainly dependent on the classical chemical processes, resulting in formation of DL-carnitine mixtures, and requires subsequent resolution of D- and L-enantiomers. Whereas L-carnitine is the natural and physiologically effective form, D-carnitine is a competitive inhibitor of L-carnitine. Thus, development of a better manufacturing process based on microbial fermentation could be of great practical significance. However, only some eukaryotic microorganisms, such as *Saccharomyces cerevisiae* and other fungal strains that are capable of accumulating L-carnitine in their cells even with a low yield, have been reported (Sigma Tau. 1983. Process for enzymatically producing L-carnitine. US patent 4371618; Nippon Pet Food. 1990. Method for preparation of L-carnitine. Japan patent 2-069188; Yakult Honsha. 1993. L-Carnitine preparation. Japan patent 5-199890), and a few other microorganisms have been known to convert γ-butyrobetaine to L-carnitine [12, 16]. Recent studies have also shown that strains belonging to genera *Enterobacteriaceae*, such as *Escherichia coli*, *Salmonella typhimurium*, and *Proteus vulgaris*, are able to biotransform crotonobetaine to L-carnitine [6, 7, 9, 14, 18].

With a long-term goal in mind, we attempted to create a genetically engineered microorganism, which entails reconstruction of the *de novo* metabolic pathway for L-carnitine biosynthesis in a favorite microorganism other than *N. crassa*. As a first step toward rebuilding the metabolic pathway for L-carnitine biosynthesis in a favorite microorganism, we screened the putative *N. crassa* cDNA clones, encoding γ-butyrobetaine hydroxylase involved in the last step of L-carnitine biosynthesis, based on the homology of known

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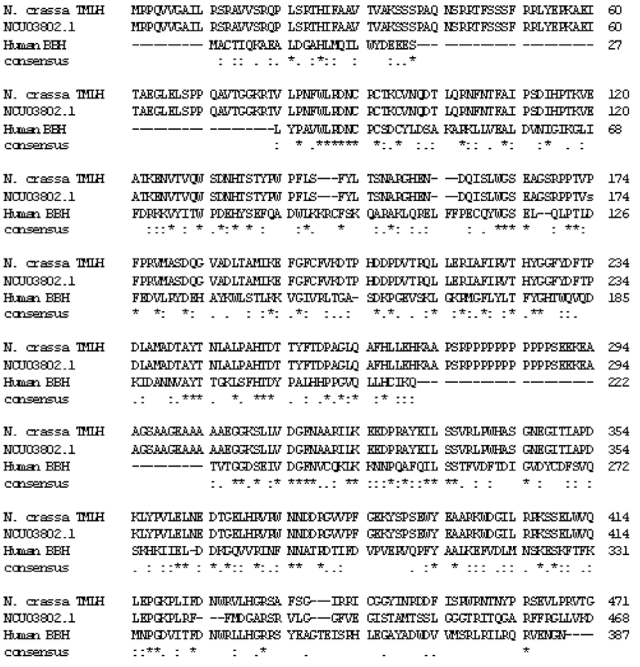

Fig. 1. Alignment of the deduced protein sequence encoded by NCU03802.1 with the amino acid sequence of *N. crassa* TMLH and human γ-butyrobetaine hydroxylase (BBH).
Amino acid sequences were aligned using the T-COFFEE program [17]. Dashes in the sequences represent gaps introduced to maximize alignment. Symbols: *, Identical residues; :, conserved residues.

human γ-butyrobetaine hydroxylase to the whole genome database of *N. crassa* (http://www.broad.mit.edu/annotation/fungi/neurospora_crassa_7/index.html). A BLASTp similarity search [1, 2, 13] revealed a set of three open reading frames (ORFs), NCU03802.1, NCU02196.1, and NCU06891.1, whose deduced amino acid sequences showed 23, 24, and 27% identity to human γ-butyrobetaine hydroxylase, respectively. These hypothetical proteins consisted of predicted coding sequences of 1,407, 1,278, and 3,786 bp, respectively. Alignment of these ORFs with the *N. crassa* genomic sequence showed that the coding sequence of these ORFs, NCU03802.1, NCU02196.1, and NCU06891.1, consisted of eight, two, and five exons, respectively. Interestingly, the deduced amino acid sequence of NCU03802.1 revealed extensive homology with ε-*N*-trimethyllysine hydroxylase (TMLH) (91% identity) (Fig. 1), suggesting that duplication of a gene segment or the entire genome might have occurred, and that the duplicated copy of a gene has been evolved to acquire a novel function in *N. crassa*. The genomic sequences of TMLH and NCU03802.1 have been mapped to different chromosomes in the genome database of *N. crassa*, and the deduced amino acid sequence of NCU03802.1 contains a unique and unusual 11 residue poly(P) region that was also found in *N. crassa* TMLH [20]. As shown in Fig. 1, the amino acid sequence of the human γ-butyrobetaine hydroxylase also showed significant homology with the *N. crassa* TMLH (25% identity) as well as the deduced amino acid sequence of NCU03802.1. When conservative substitutions were taken into account, the homology of the human γ-butyrobetaine hydroxylase to the deduced amino acid sequences of NCU03802.1 and the *N. crassa* TMLH was 49 and 50%, respectively. In addition, the deduced amino acid sequence of NCU03802.1 also showed significant homology with other γ-butyrobetaine hydroxylases from *Mesorhizobium loti*, *Pseudomonas* sp. strain AK-1, *Candida albicans* SC5314, and *Caenorhabditis elegans* (data not shown). Based on this result, the predicted coding sequences (NCU03802.1, NCU02196.1, and NCU06891.1) were used to design primers 3802.1F and 3802.1R, 2196.1F and 2196.1R, and 6891.1F and 6891.1R, respectively (Table 1), overlapping with the putative start and stop codons. These primers were used to amplify the ORFs from a *N. crassa* cDNA yeast expression library (Lambda g15-NC cDNA; Fungal Genetic Stock Center, Kansas City, KS, U.S.A.) by PCR. Fifty μl of PCR reaction mixture contained 10 μl of 10× reaction buffer [100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-Cl (pH 8.75), 20 mM $MgSO_4$, 1% Triton X-100, 1,000 μg/ml BSA], 200 μM each of dNTP, 100 pmol each of primers, 2.5 units of *Top-Pfu* DNA polymerase

Table 1. Oligonucleotides used in this study.

Oligonucleotide	Sequence[a]	Restriction site
3802.1F	5'-attccgatatc catATGAGACCGCAAGTGGTAGG-3'	EcoRV and NdeI
3802.1R	5'-ccgatgaattcTCAGTCCTTAACCAGTAACCC-3'	EcoRI
2196.1F	5'-actgataagcttcATGGCCACGGCAGCGGTTCAGG-3'	HindIII
2196.1R	5'-ccgatgaattcTCAATACCCTCCCCCACCCTGCG-3'	EcoRI
6891.1F	5'-attccgatatcATGGGGTTCCTCGCTACTCTCATCG-3'	EcoRV
6891.1R	5'-attccgatatcTTATGCGTTCCAGTTCACCGTGCC-3'	EcoRV
P1	5'-taggtaccACGCGCATAACCGCTAGAGTA-3'	KpnI
P2	5'-tactcgagTGAGATAGTTGATTGTATGCTTGG-3'	XhoI
T1	5'-tcactagtCGTTGTTGACACTTCTAAATAAGCG-3'	SpeI
T2	5'-tagagctcTACCAGGATAGGACACGGTGTT-3'	SacI

[a]The restriction site is underlined and upper case letters refer to the sequences of the corresponding gene.

(Solgent, Daejeon, Korea), and 250 ng of Lambda g15-NC cDNA as the template. The samples were placed in the PTC-100 Programmable Thermal Controller (MJ Research, Waltham, MA, U.S.A.), and the thermal cycling program consisted of an initial step at 95°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 56°C, and 5 min at 72°C. A final extension step of 7 min at 72°C was included. Using the *N. crassa* cDNA library as the template, PCR products of approximately 1,407 and 1,278 bp, containing the putative ORF of NCU03802.1 and NCU02196.1 cDNA, were successfully amplified with primers 3802.1F and 3802.1R, and 2196.1F and 2196.1R, respectively, whereas no PCR products were obtained with primers 6891.1F and 6891.1R. These putative ORF sequences were cloned into the *S. cerevisiae*-*E. coli* shuttle vector pRS426 [8] between the glucose-inducible *adh1* promoter and the *adh1* transcriptional terminator, and used directly to transform *S. cerevisiae* SJ7164 (*his3-delta1*, *leu2-3*, *leu2-112*, *ura3-52*, *trp1-289*) with the lithium acetate method [10, 14]. In detail, a 990-bp *adh1* promoter region and 706-bp terminator region were first amplified using primers P1 and P2, and T1 and T2, respectively, and genomic DNA from *S. cerevisiae* SJ7164 as the template. The resulting 990-bp products containing the sequence of the *adh1* promoter were digested with KpnI and XhoI, and subcloned into the corresponding sites of pBluescript II KS+ (Stratagene, La Jolla, CA, U.S.A.). The resulting plasmid was digested with SpeI and SacI, and ligated with 706-bp PCR products containing the sequence of the *adh1* terminator that had also been digested with SpeI and SacI. The resulting plasmid, designated pSJ400, was digested with EcoRV and EcoRI, and ligated with a 1,407-bp EcoRV-EcoRI PCR fragment containing the putative ORF of NCU03802.1 cDNA to generate the plasmid pSJ401. A KpnI-SacI fragment from pSJ401 containing the putative ORF of NCU03802.1 cDNA between the *adh1* promoter and the *adh1* terminator was inserted into the corresponding sites of pRS426 to generate the plasmid pSJ420. To construct the expression plasmid overexpressing the hypothetical protein of NCU02196.1, an internal HindIII-EcoRI fragment of pSJ420 was replaced with a 1,278-bp PCR fragment containing the putative ORF of NCU02196.1 cDNA digested with HindIII-EcoRI to obtain pSJ404. The sequencing results revealed the expected ORFs of 1,407 bp encoding a protein of 468 amino acids for NCU03802.1, and 1,278 bp encoding a protein of 425 amino acids for NCU02196.1 (data not shown).

In the subsequent experiments, these hypothetical proteins (NCU03802.1 and NCU02196.1) were expressed in *S. cerevisiae* strain SJ7164 grown on glucose minimal medium (6.7 g of yeast nitrogen base without amino acids, 20 g of glucose, and 20 mg of uracil per liter), and γ-butyrobetaine hydroxylase activity in the crude cell-free extract of yeast transformants, containing the *N. crassa* ORFs for the putative γ-butyrobetaine hydroxylase, was measured by a procedure in which L-carnitine produced by the hydroxylase was measured colorimetrically [23, 25]. Crude cell-free extracts for γ-butyrobetaine hydroxylase activity assays were obtained from an exponentially growing culture. The reaction mixture in a final volume of 500 μl was composed of 20 mM potassium phosphate buffer, pH 7.0, containing 20 mM KCl, 3 mM α-ketoglutarate, 10 mM sodium ascorbate, 2 g/l Triton X-100, 0.25 mM $(NH_4)_2Fe(SO_4)_2$, and 0.2 mM γ-butyrobetaine. The amount of L-carnitine produced by γ-butyrobetaine hydroxylase was determined and calculated from a standard curve obtained by using a known amount of L-carnitine. As shown in Table 2, γ-butyrobetaine hydroxylase activity in the crude cell-free extracts of transformants that were expected to overexpress the protein product of NCU03802.1 (pSJ420) showed high γ-butyrobetaine hydroxylase activity, whereas no γ-butyrobetaine hydroxylase activity was detected in the extracts of transformants expected to overexpress the protein product of NCU02196.1 (pSJ404). It was found that γ-butyrobetaine hydroxylase activity needed an exogenous substrate, γ-butyrobetaine, since the formation of L-carnitine in the strain SJ7164 containing pSJ420 was γ-butyrobetaine-dependent. Additionally, the activity in the extracts of transformants containing pRS426 (negative control) was virtually undetectable even in the presence of exogenous γ-butyrobetaine, indicating that the *S. cerevisiae* strain used in this study lacks γ-butyrobetaine hydroxylase activity. These results suggest that the protein product of NCU03802.1 functions as a γ-butyrobetaine hydroxylase involved in the last step of the L-carnitine biosynthesis pathway of *N. crassa*. Our future work will be focused on verifying the physiological relevance of NCU03802.1 under conditions where L-carnitine-dependent metabolic activity of a *S. cerevisiae* mutant strain on non-fermentable carbon sources [19, 22] is restored by γ-butyrobetaine hydroxylase activity *in vivo*.

Table 2. Enzyme activity measured in the extracts of yeast transformants containing the *N. crassa* ORFs for the putative γ-butyrobetaine hydroxylase.

Host	Plasmid	Specific activity[a] (μmol/min/mg protein)
S. cerevisiae SJ7164	pRS426	ND[b]
	pSJ404	ND
	pSJ420	0.036±0.003

[a]Protein concentrations in the extracts were determined by the method described previously [4], using bovine serum albumin as a standard. Data represent means of three independent measurements including the standard deviation.
[b]ND, not detected.

The GenBank accession numbers of the sequences, NCU03802.1 and NCU02196.1, reported in this paper are XM956098 and XM954497, respectively.

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