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Cloning and Characterization of *Mannheimia succiniciproducens* MBEL55E Phosphoenolpyruvate Carboxykinase (*pckA*) Gene

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Abstract A *pckA* gene encoding phosphoenolpyruvate carboxykinase (PEPCK) was cloned and sequenced from the succinic acid producing bacterium *Mannheimia succiniciproducens* MBEL55E. The gene encoded a 538 residue polypeptide with a calculated molecular mass of 58.8 kDa and a calculated pI of 5.03. The deduced amino acid sequence of the *M. succiniciproducens* MBEL55E PEPCK was similar to those of all known ATP-dependent PEPCKs.

Keywords: phosphoenolpyruvate carboxykinase, *pckA*, *Mannheimia succiniciproducens* MBEL55E

Phosphoenolpyruvate carboxykinases (PEPCKs, EC 4.1.1.49) catalyze the decarboxylation and phosphorylation of oxaloacetate to form phosphoenolpyruvate (PEP) and play an important role in the gluconeogenic pathway in most organisms. PEPCKs have been classified according to their specificities for nucleotides, GTP and ATP. The enzymes from mammals and a variety of eukaryotes have a specificity for GTP, while the enzymes from bacteria, yeast and plants have a specificity for ATP [1]. PEPCKs also have different quaternary structures: the GTP-specific enzymes are generally monomers, while the ATP-specific enzymes can be monomers or oligomers [1,2]. However, all PEPCKs are known to have the absolute requirement for a divalent metal ion, such as Mn^{2+}, Mg^{2+} or Co^{2+}, as supported by the existence of two metal binding domains [3,4].

PEPCKs can have different physiological roles as a gluconeogenic or an anaplerotic enzyme. In most organisms ranging from prokaryotes to vertebrates, PEPCKs are key enzymes in gluconeogenesis. The growth of *Escherichia coli* on C_4 substrates and growth of *Saccharomyces cerevisiae* on ethanol are significantly affected by the presence of their respective PEPCKs [5-8]. In capnophilic anaerobic bacteria isolated from animal rumen, intestines, and mouths, PEPCKs function as a CO_2-fixing enzyme [9-11]. Some of these capnophilic bacteria produce succinic acid as a fermentation end-product based on a series of reaction steps involving the carboxylation of PEP by PEPCKs [10]. Others produce propionic acid as a end-product using intermediate succinic acid [12]. Recently, we have isolated a novel succinate-producing bacterium *Mannheimia succiniciproducens* MBEL55E from the rumen of a Korean cow, which was deposited at the Korean Collection for Type Cultures (Daejeon, Korea) as KCTC 0769BP. *M. succiniciproducens* MBEL55E is a metabolically versatile bacterium capable of growing either anaerobically and aerobically. It produces acetic acid, formic acid, lactic acid, and ethanol as well as succinic acid depending on the growth conditions. The growth rate of *M. succiniciproducens* MBEL55E and succinic acid formation are regulated by the level of CO_2 in the culture medium. Under CO_2 rich condition, the growth rate of *M. succiniciproducens* MBEL55E was enhanced, and the carbon flux was directed toward succinic acid formation. Whereas, under CO_2 poor condition, the growth rate was lower, and the carbon flux was directed toward lactic acid formation (unpublished results). In addition, *M. succiniciproducens* MBEL55E is a facultative anaerobe and can grow aerobically. Interestingly, under O_2 rich condition, it produced lactic and acetic acids as main products irrespective of the initial glucose concentration. These results suggest that PEPCK also plays an important role in the succinic acid production by *M. succiniciproducens* MBEL55E.

Most of the ATP-dependent PEPCK encoding genes that have been cloned from prokaryotes are the enzyme responsible for gluconeogenesis (*E. coli* [13], *Rhizobium meliolti* [14], *Rhizobium* sp. [15], *Staphylococcus aureus* [16], and *Rhodopseudomonas palustris* [17]). To date, however, among succinic acid producing bacteria, only the ATP-dependent PEPCK-encoding gene of *Anaerobiospirillum succiniciproducens* has been cloned and characterized [18]. *A. succiniciproducens* is a metabolically versatile organism like *M. succiniciproducens* MBEL55E [19]. In both bacteria, PEPCK seems to be the key enzyme in central metabolic pathway and plays a key role in cell growth and redirecting the carbon flux. In this paper, we report on the cloning and structural analysis of the *pckA* gene from the novel succinic acid producing bacterium *M. succiniciproducens* MBEL55E.

M. succiniciproducens MBEL55E (KCTC 0769BP) was the source of the chromosomal DNA for the construc-

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tion of the genomic library. *M. succiniciproducens* MBEL55E was grown in sealed anaerobic bottles containing 100 mL of a complex medium containing 5 g/L glucose, 2.5 g/L polypeptone, 2.5 g/L yeast extract, 3 g/L K_2HPO_4, 2 g/L NaCl, 2 g/L $(NH_4)_2SO_4$, 0.2 g/L $CaCl_2{\cdot}2H_2O$, 0.4 g/L $MgCl_2{\cdot}6H_2O$ and 5 g/L $MgCO_3$ with CO_2 as the gas phase. *E. coli* XL1-Blue (Stratagene Cloning Systems, La Jolla, CA, USA) was used to construct the genomic library and used for the subclonings. *E. coli* cultures were grown at 37°C in Luria-Bertani medium [20]. Plasmid vector was pUC19 (Sigma, St. Louis, MO, USA). Ampicillin was added at 100 µg/mL.

M. succiniciproducens MBEL55E chromosomal DNA was purified using ethanol precipitation method [20] and partially digested with the restriction enzymes *Bam*HI, *Eco*RI, *Pst*I, and *Hind*III. Appropriate DNA fragments (2 to 6 kb) were isolated from a 10 to 40% sucrose gradient and ligated into bacterial alkaline phosphatase treated pUC19. *E. coli* was transformed with the ligation mixture by electroporation.

Plasmid DNA purification, restriction analysis, PCR, and colony and DNA hybridizations were performed by conventional techniques [20]. DNA was recovered from agarose gels with the Geneclean II kit (BIO 101, La Jolla, CA, USA). All PCRs were carried out in a Perkin-Elmer DNA thermal cycler (Model 480, Foster City, CA, USA) according to the manufacturer's instructions. For the amplification of homologous probes for the *pckA* gene of *M. succiniciproducens* MBEL55E by PCR, degenerate primers were synthesized. The oligonucleotides used in this study were synthesized by the Genotech Co. (Daejeon, Korea). Based on the amino acid sequences of a highly conserved domain of PEPCK proteins, degenerate primers #1 (5'-GGYCTKTCMGGCACYGGTAARACC-3'), #2 (5'-CTSATYGGYGAYGAC-3'), #3 (5'-ATYAASCTSTCKAARGAA-3'), #4 (5'-TTCYTTMGASAGSTTRAT-3') and #5 (5'-GGTATCYTTAATSGAGATACG-3') were designed. The PCR fragments were isolated, cloned into pUC19 vector, and were sequenced.

The DNA sequence of the cloned gene was deter-

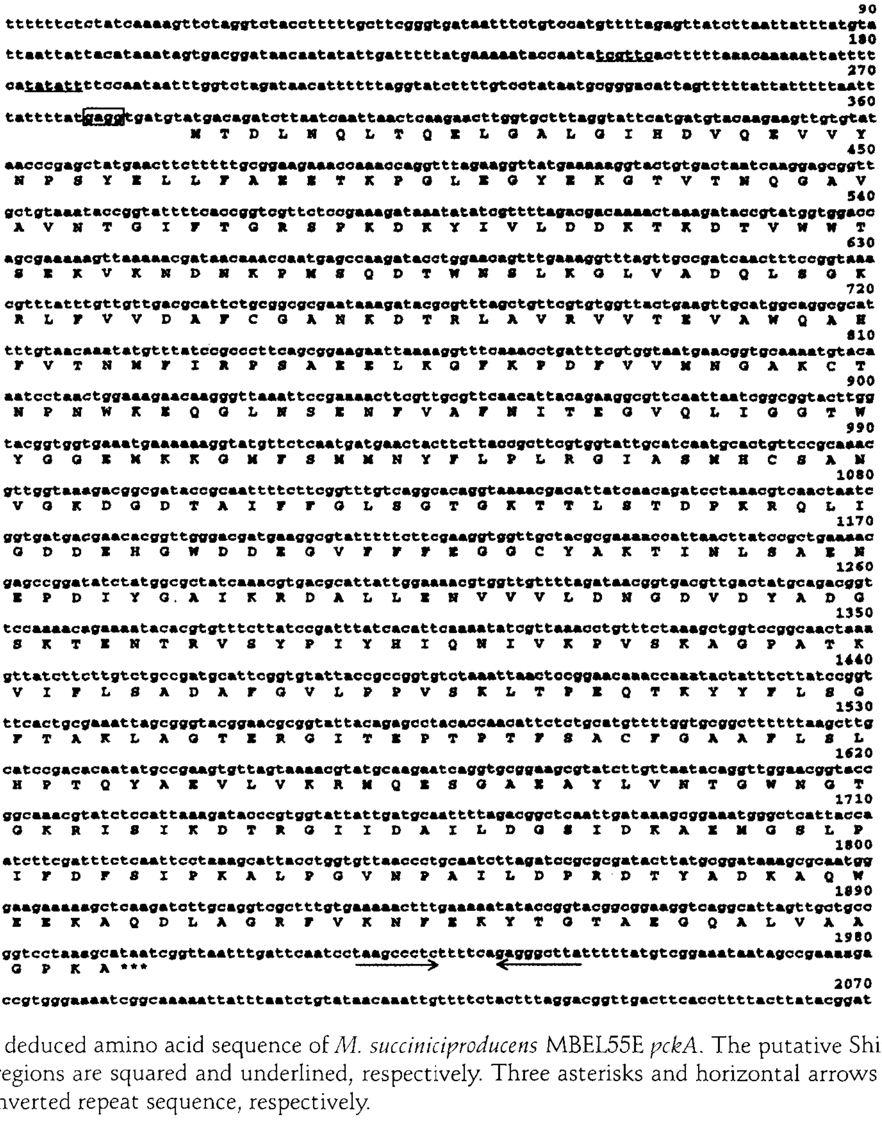

Fig. 1. Nucleotide and deduced amino acid sequence of *M. succiniciproducens* MBEL55E *pckA*. The putative Shine-Dalgarno (SD) and putative –35 and –10 regions are squared and underlined, respectively. Three asterisks and horizontal arrows indicate a stop codon and a terminator-like inverted repeat sequence, respectively.

mined on both strands starting with the universal and reverse primers of pUC19 followed by additional primers designed after obtaining further sequence information. The automated fluorescence sequencer (ABI Prism model 377, Perkin Elmer Co., IL, USA) and the sequencing kit were used. Sequence analysis was performed using the DNASIS software package (Hitachi Co., Tokyo, Japan) and the BLAST programs of the National Center for Biotechnology Information (Bethesda, MD, USA). A phylogenetic tree was built using the DNASIS software package.

Based on the amino acid sequences of the highly conserved domains of PEPCK proteins [1], five oligonucleotides primers (see Materials and Methods) were designed in order to amplify a portion of the *pckA* gene, and subsequently to use it as a homologous probe. Using the degenerate primers and *M. succiniciproducens* MBEL55E chromosomal DNA as a template, a 550 bp PCR product was obtained. The PCR product was subsequently cloned into pUC19, sequenced, and its deduced amino acid sequence was analyzed by BLAST. The sequence analysis showed that the deduced amino acid sequence of the PCR product had greater than 70% homology to parts of the *pckA* gene products of prokaryotes and eukaryotes (data not shown).

The PCR product was used as a probe to screen the *M. succiniciproducens* MBEL55E genomic library. Screening of 4,000 clones yielded 15 positive clones. Restriction analysis showed that all positive clones contained the recombinant plasmids with ca. 3.5 kb inserted DNA. The inserted DNAs were used as probes in a dot blotting and southern blotting of *M. succiniciproducens* MBEL55E chromosomal DNA digested with various restriction enzymes. The strong hybridization signals obtained indicated that the cloned gene originated from *M. succiniciproducens* MBEL55E.

The 3.5 kb *Pst*I-*Pst*I inserted DNA was sequenced and analyzed. Analysis of all possible translation frames of the complete inserted DNA fragment showed one 1,614 bp open reading frame (ORF). It encoded a 538 residue polypeptide (a calculated molecular mass of 58.8 kDa) with an ATG start codon at position 289 (Fig. 1). The ATG was preceded by a potential ribosome binding site, GAGG, located 7 bp upstream. An inverted repeat sequences (10 bp), forming a G+C rich stem-loop, was present downstream of the stop codon (TAA) followed by at least five T residues, the characteristics of a *rho*-independent terminator [21]. The BLAST search of the ORF suggested that the cloned gene encondes PEPCK.

The codon frequency of the *M. succiniciproducens* MBEL55E *pckA* was compared with that of the *A. succiniciproducens pckA* gene as well as the *E. coli pckA* gene. The overall G+C content of the *M. succiniciproducens* MBEL55E *pckA* gene was 41.6%, which was relatively lower than that of the *A. succiniciproducens pckA* gene (47.9%) and that of the *E. coli pckA* gene (53.7%). Strong codon preferences for a few amino acids were found in *M. succiniciproducens* MBEL55E. First, the *M. succiniciproducens* MBEL55E *pckA* absolutely prefers AAA (100%) for lysine codon, whereas the *E. coli pckA* and the *A. succiniciproducens pckA* prefer AAA (75%) and AAG (96%), respectively. Second, 94% of *M. suciniciproducens* MBEL55E *pckA* glutamate codons are GAA, whereas those of the *E. coli pckA* and *A. succiniciproducens pckA* are GAA (73%) and GAG (76%), respectively. Third, in the case of glutamine codon, CAG (88%) codons are preferred by the *M. succiniciproducens* MBEL55E *pckA* , while CAG (79%) codons are preferred by the *E. coli pckA* and CAG (100%) codons are used by the *A. succiniciproducens pckA* . Finally, 76% of the *M. succiniciproducens* MBEL55E *pckA* glycine codons are GGT, while *E. coli* and *A. succiniciproducens* prefer GGC (53%) and GGC (61%), respectively.

The pI value of *M. succiniciproducens* MBEL55E PEPCK was estimated for the deduced amino acid sequence to be 5.03, and was similar to those of other bacterial

Table 1. Conservation of the active site residues of ATP-dependent PEPCKs. Identical residues are indicated with asterisks below the sequence alignment

	PEPCK-specific domain	Kinase-1a	Kinase-2a	Covalent metal Ion binding site	Ribose binding site	Adenine binding site
M. succiniciproducens	$\mathrm{GTWYGGEMKK}_{211}$	$\mathrm{GLSGTGKT}_{253}$	LIGDD_{267}	$\mathrm{GVFFFEGG}_{282}$	$\mathrm{KTINLSAENE}_{295}$	$\mathrm{RISIKDT}_{453}$
T. brucei	GTEYAGEMKK	GLSGTGKT	LIGDD	GVFNIEGG	KAIGLNPETE	RMPLRVT
H. influenzae	GTWYGGEMKK	GLSGTGKT	LIGDD	GIFNFEGG	KTIHLSEENE	RISIKDT
A. succiniciproducens	NTWYGGEMKK	GLSGTGKT	LIGDD	GVFNFEGG	KVINLSKENE	RISIKDT
R. palustris	GTQYAGEMKK	GLSGTGKT	LIGDD	GVFNFEGG	KCIKLSAENE	RMPIKVT
S. cerevisiae	GTEYAGEMKK	GLSGTGKT	LIGDD	GVFNIEGG	KCINLSAEKE	RCPLKYT
E. coli	GTWYGGEMKK	GLSGTGKT	LIGDD	GVFNFEGG	KTIKLSKEAE	RISIKDT
C. sativus	GTQYAGEMKK	GLSGTGKT	LIGDD	GVSNIEGG	KCIDLSREKE	RIKLAYT
R. meliloti	GTSYAGEMKK	GLSGTGKT	LIGDD	GVFNFEGG	KAIRLSEAAE	RMPIKVT
U. panicoides	GTQYAGEMKK	GLSGTGKT	LIGDD	GVSNIEGG	KCIDLSKEKE	RIKLPYT
T. cruzi	GTETAGEMKK	GLSGTGKT	LIGDD	GVFNIEGG	KAIGLNPKTE	RMPLRVT
S. aureus	GTETAGEMKK	GLSGTGKT	LIGDD	GVFNIEGG	KAINLSKEKE	RISLHYT
Rhizobium sp.	GTWYAGEMKK	GLSGTGKT	LIGDD	GVFNFEGG	KAIRLSEAAE	RMPIKVT
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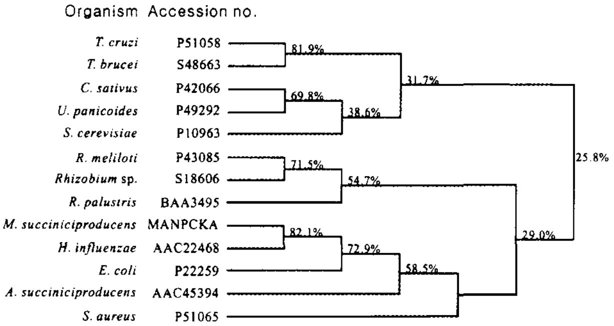

Fig. 2. Phylogenetic tree of the known ATP/ADP-dependent PEPCKs.

PEPCKs. The amino acid sequences of PEPCKs including that of *M. succiniciproducens* MBEL55E were aligned, and important domains are shown in Table 1. It is clear that the important domains are highly conserved in all PEPCKs. Phylogenic analysis showed that the *M. succiniciproducens* MBEL55E PEPCK was more closely related to the ATP dependent PEPCKs from gram-negative bacteria than gram-positive bacteria and eukaryotes (Fig. 2). *M. succiniciproducens* MBEL55E PEPCK contained all of the functional residues conserved in all ATP dependent PEPCKs (Table 1). The phosphate-binding and adenine-binding consensus sites of ATP-dependent PEPCKs were identified in highly conserved regions [22]. A divalent or transition metal ion binding (G----EGG) site consensus sequence could also be determined by homology [23]. These results suggested that the *M. succiniciproducens* MBEL55E PEPCK is an ATP-dependent enzyme, which may function in a similar way to that of *A. succiniciproducens* for the production of succinic acid.

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