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Cloning, Sequencing, and Overexpression of the *Anaerobiospirillum succiniciproducens* Phosphoenolpyruvate Carboxykinase (*pckA*) Gene

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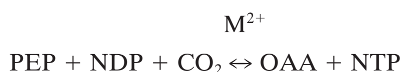
The phosphoenolpyruvate (PEP) carboxykinase-encoding gene from the anaerobic, CO_2-fixing, succinate-producing bacterium *Anaerobiospirillum succiniciproducens* was cloned, sequenced, and expressed in *Escherichia coli*. The gene encoded a 532-residue polypeptide with a calculated molecular mass of 58.7 kDa. The sequence of the *A. succiniciproducens* PEP carboxykinase was similar to those of all known ATP/ADP-dependent PEP carboxykinases. In particular, the *A. succiniciproducens* enzyme was 67.3% identical and 79.2% similar to the *E. coli* enzyme. The *A. succiniciproducens pckA* transcription start site was determined, and putative promoter regions were identified. The recombinant enzyme was overexpressed in *E. coli*. The purified enzyme was indiscernible from the native enzyme by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and had the same activity as the native enzyme.

Phosphoenolpyruvate carboxykinase (PCK) (EC 4.1.1.49) catalyzes the reversible carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate (OAA):



$$PEP + NDP + CO_2 \overset{M^{2+}}{\leftrightarrow} OAA + NTP$$

(30) (where NDP and NTP stand for nucleoside diphosphate and triphosphate, respectively, and M^{2+} designates a divalent transition metal ion). Adenosine nucleotides are mainly used by microbial and plant enzymes, but enzymes from higher organisms do use guanosine or inosine phosphates. The absolute requirement for a metal ion by PCKs is well documented (5, 30): two metal-binding sites have been described, one binding a cation-nucleotide complex and the other binding a free divalent cation (3). Typically, Mn^{2+} can, by itself, fulfill the metal ion requirement, but Mg^{2+} or Co^{2+} can be substituted—with reduced activity—for the Mn^{2+} ion (30).

PCK has been shown to physiologically work in both directions. (i) It is a key gluconeogenesis enzyme in organisms ranging from prokaryotes to vertebrates (30). Changing *Saccharomyces cerevisiae* from growing on ethanol to growing on glucose is accompanied by a rapid disappearance of cellular PCK activity (9, 20). PCK is important for *Escherichia coli*'s growth on C_4 substrates (10), and it has been shown at low expression levels to control the growth rate of succinate-grown gluconeogenic *E. coli* (4). (ii) In animal rumen-, intestine-, and mouth-colonizing anaerobic bacteria that require CO_2 to grow, PEP carboxykinase functions as a CO_2-fixing enzyme. Some of these bacteria form the fermentation end product, succinate (13). Others metabolize succinate (an intermediate step) via succinyl-, methylmalonyl-, and propionyl-coenzyme A to form propionate as the fermentation end product (16). The anaerobic, gram-negative bacterium *Anaerobiospirillum succiniciproducens* produces up to 35 g of succinate per liter (7). It also produces acetate, lactate, ethanol, and formate in concentrations that depend on growth conditions. *A. succiniciproducens*'s growth rate and succinate-versus-lactate formation are regulated by the culture's CO_2 concentration and pH. In conditions favoring succinate production (pH 6.2 and high CO_2 concentration), 65% of the glucose carbon used by *A. succiniciproducens* is directed toward succinate production (from PEP, by CO_2 fixation) (26). Under these conditions, PCK expression increases significantly (it represents up to 10% of whole-cell protein), and high PCK levels can be detected. Samuelov et al. (26) proposed that PCK played a key role in succinate production by fixing CO_2 to form OAA. *A. succiniciproducens* PCK was purified and characterized as described previously (24).

Most of the ATP/ADP-dependent PCK-encoding genes that have been cloned, including four from prokaryotes (*E. coli* [18], *Rhizobium meliloti* [22], *Rhizobium* sp. strain NGR234 [21], and *Staphylococcus aureus* [28]), are gluconeogenesis enzymes. To date, *A. succiniciproducens*'s enzyme is the best-characterized ATP/ADP-dependent, catabolic, and CO_2-fixing PCK. To learn more about this enzyme, we chose to clone and sequence its gene to study its relationship to decarboxylating ATP/ADP-dependent PCKs. After overexpressing the *A. succiniciproducens pckA* gene to generate sufficient enzyme for structure-function studies, we also compared the *A. succiniciproducens* PCK's kinetic properties to those of decarboxylating ATP/ADP-dependent PCKs.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *A. succiniciproducens* (ATCC 29305) was the source of chromosomal DNA for construction of the genomic library. *E. coli* ER2309 (New England Biolabs, Beverly, Mass.) (13a) was used to construct the library, and *E. coli* DH5α (Life Technologies, Gaithersburg, Md.) was used for the subclonings and gene expression. Plasmid vectors were pUC18 (Pharmacia, Piscataway, N.J.), pBR322 (Pharmacia), and pProEX-1 (Life Technologies). Figure 1 shows the plasmids constructed in this study. *A. succiniciproducens* was grown as described previously (26). *E. coli* cultures were grown in Luria-Bertani medium (2). Ampicillin and chloramphenicol were added at 100 and 34 μg/ml, respectively.

Library construction. *A. succiniciproducens* chromosomal DNA was purified as described previously (19) and partially digested with the restriction enzyme *Sau*3AI. Two- to 6-kb fragments were isolated from a 10 to 40% sucrose gradient (2) and ligated into *Bam*HI-bacterial alkaline phosphatase-treated pUC18. *E. coli* ER2309 was transformed with the ligation mixture by electroporation.

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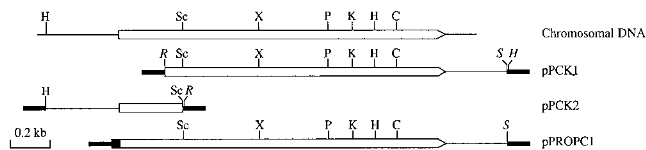

FIG. 1. Physical and genetic maps of the plasmids constructed in this study. Plasmid pPCK3 is not shown; it differs from pPCK1 only by the absence of the *Eco*RI restriction site. Open arrows, *pckA* gene; thin lines, noncoding *A. succiniciproducens* DNA; thick lines, vector DNA. Sc, *Sac*I; S, *Sal*I; X, *Xho*I; P, *Pvu*II; K, *Kpn*I; H, *Hin*dIII; C, *Cla*I; R, *Eco*RI.

DNA manipulations. Plasmid DNA purification, restriction analysis, PCR, and colony and DNA hybridizations were performed by conventional techniques (2, 25). DNA was recovered from agarose gels with the Geneclean II kit (BIO 101, La Jolla, Calif.). The oligonucleotides used in this study were synthesized at the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University. Primers 1 (5′-ACATGCATGCNAARTAYGGNATHACNGGNGCNAC-3′ [encoding peptide AKYGITGAT]) and 2 (5′-CGYTTDATNGCNCCCCADATRTC-3′ [encoding peptide DIWGAIKR]) were used to synthesize the homologous probe for colony hybridization. Ligation-mediated PCR was performed with the PCR in vitro cloning kit (Pan Vera; Madison, Wis.). Primers S1 and S2 were 5′-TCTTCATCTCACCACCGTACCA-3′ and 5′-CTTTGAAGCGTTCAGAACTACG-3′, respectively.

Primer extension. Total *A. succiniciproducens* RNA was purified from a late-exponential-growth-phase culture as described previously (14). Primer extension (primer 4; 5′-GTTGGTAGCACCAGTAATGCCG-3′) was performed as described previously (2).

Nucleotide sequence determination and sequence analysis. Sequences were determined on both strands by the dideoxy chain termination technique (27) with the Sequenase, version 2.0, kit (U.S. Biochemical Corp., Cleveland, Ohio). PCR products were sequenced with the Cyclist Exo$^-$ Pfu DNA sequencing kit (Stratagene, La Jolla, Calif.). Sequencing data were analyzed with the Wisconsin Sequence Analysis Package, version 8.0 (Genetics Computer Group, Madison, Wis.) (8). The dendrogram shown in Fig. 4 was built by doing progressive, pairwise sequence alignments and clusterings (program PileUp, available through the Genetics Computer Group).

For purposes of comparison, the *E. coli pckA* sequence was corrected in two steps. (i) The 466-residue incorrect protein sequence was aligned with the correct sequence (obtained from the crystal structure) (SwissProt, accession no. p22259). Unambiguous corrections were made by hand in the 5′-terminal part of the gene to match the *pckA* translation product with the correct sequence. (ii) The 3′ end (306 nucleotides [nt]) was obtained by screening GenBank with the *pckA* sequence. A single truncated open reading frame (ORF) was found in the reverse orientation downstream of the *E. coli ompB-envZ* genes (GenBank accession no. J01656). This truncated ORF encoded a 102-residue product 100% identical to *E. coli* PCK's true C terminus. We assumed that this truncated ORF corresponded to the *E. coli pckA* 3′ end.

Enzyme purification, assay, and digestion. Native *A. succiniciproducens* PCK was purified as described previously (24). Enzyme assays were performed as described previously (24), except that the reaction mixture contained both $MnCl_2$ (5 mM) and $MgCl_2$ (10 mM), and the carboxylation and decarboxylation assays were performed at pHs 6.5 and 6.8, respectively. Activities were corrected for nonenzymatic OAA decarboxylation. The recombinant enzyme was expressed with the pProEX-1 protein expression system (Life Technologies) and purified with a Ni-nitriloacetic acid (Ni-NTA) affinity resin. The His tag and linker sequences were deleted with tobacco etch virus (TEV) protease (Life Technologies) (23). Protein concentrations were determined with the Bio-Rad protein assay kit (Bio-Rad, Richmond, Calif.) with bovine serum albumin as the standard. Native PCK was digested with *Pseudomonas fragi* Asp-N endoproteinase (Sigma, St. Louis, Mo.); the peptides were separated on a high-performance liquid chromatography C_{18} phase-coated silica column (0.8 by 250 mm) (LC Packings, Switzerland). Peptide N-terminal sequence analyses were performed by the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University.

Nucleotide sequence accession number. The GenBank accession number for the sequence published in this article is U95960.

RESULTS

Cloning of the PEP carboxykinase gene. The *pckA* gene was cloned by homologous colony hybridization. The N-terminal sequences of internal peptides obtained by proteolytic digestion of the native *A. succiniciproducens* PCK were used to design degenerate oligonucleotide primers and to synthesize a homologous probe by PCR. Some of the peptide sequences obtained after the digestion of *A. succiniciproducens* PCK with *P. fragi* Asp-N endoproteinase could be reverse translated into low-degeneracy oligonucleotides (Fig. 2). Using primers 1 and 2 with *A. succiniciproducens* chromosomal DNA as the template, we obtained a 900-bp PCR product. This PCR product was used as a probe to screen the *A. succiniciproducens* genomic library. Of the 2,500 clones, 12 hybridized with the probe. These 12 clones contained recombinant plasmids that carried 1.4- to 1.7-kb DNA inserts. All 12 inserts contained an overlapping region with conserved *Pvu*II, *Kpn*I, and *Hin*dIII restriction sites (Fig. 1). Preliminary sequencing data indicated that these inserts contained only the 3′ end of *A. succiniciproducens pckA*. Plasmid pPCK1 (Fig. 1) contained the 3′-terminal 1,371 bp of *pckA*. Four *Sau*3AI sites are clustered in the 5′ region of the pPCK1 insert (not shown) and may explain why only the 3′ end of the gene was cloned. The pPCK1 insert was used as a probe against a Southern blot of *A. succiniciproducens* chromosomal DNA digested with various restriction enzymes. The strong hybridization signals that were obtained indicated that the cloned gene originated from *A. succiniciproducens* (data not shown).

The *pckA* gene's 5′ end was cloned by ligation-mediated PCR. *Hin*dIII-digested *A. succiniciproducens* chromosomal DNA was the template. Primers S1 and S2 (complementary to non-coding strand sequences, located between the unique *Sac*I and *Hin*dIII sites in *pckA*) were used to selectively amplify the 1.6-kb *Hin*dIII chromosomal fragment carrying *pckA*'s 5′ end. A 900-bp PCR product was obtained. Sequencing showed that this product overlapped the sequenced, truncated *pckA* gene. The PCR product was digested with *Hin*dIII and *Sac*I, and a 700-bp *Hin*dIII-*Sac*I fragment was cloned in pUC18. Six clones were sequenced: five of them were identical; the sixth contained a one-base substitution. The most abundant sequence was considered to be the correct sequence; this clone was designated pPCK2 (Fig. 1).

Nucleotide sequence of the *pckA* gene. The 1.7-kb *Sac*I-*Sau*3AI insert of plasmid pPCK1 and the 700-bp *Hin*dIII-*Sac*I insert of plasmid pPCK2 were sequenced. One 1,596-nt ORF was detected (Fig. 2). It encoded a 532-residue polypeptide (calculated molecular mass, 58.7 kDa). The sequence GGAGA, located five bases upstream of the ATG start codon, probably corresponds to the Shine-Dalgarno sequence. Transcription terminator-like inverted repeats were found 41 bp downstream of the translation stop codon (Fig. 2). N-terminal sequencing of the native enzyme (24) indicated that the N-terminal methionine is probably deleted after translation in *A. succiniciproducens*. In this study, however, the N-terminal sequences of the native enzyme and of the putative product (Fig. 2) differed from the N-terminal sequence published by Podkovyrov and Zeikus (24) by one residue (Lys9 instead of Asn9). The internal peptidic sequences determined during the cloning process were identified in the putative PckA product (Fig. 2), confirming that we had, indeed, cloned the *A. succiniciproducens* gene. The overall G+C content of the *pckA* gene was 47.9%, slightly higher than the 44% genomic content reported for the *A. succiniciproducens*-type strain.

Codon usage in *A. succiniciproducens pckA*. Table 1 compares the *A. succiniciproducens pckA*'s codon frequency with the *E. coli pckA*'s codon frequency as well as with the average codon usage in *E. coli* genes. The codon usage in *E. coli pckA* is similar to the average codon usage in *E. coli* genes. The *A. succiniciproducens pckA* G+C content (47.9%) is close to the G+C content in the *E. coli* genome (52%). While the overall codon frequency in *A. succiniciproducens pckA* is close to the *E. coli pckA* and average *E. coli* genes' codon usages, there are striking differences for a few amino acids: 62% of *A. succini-*

```
                                                                                          90
aaaaagggtagaatctttttttaaattatatttaaggcatttgtagcgaagattatgtcctctttgagggcggttagggcactaaactac
                                                                                         180
cagctgtgacgcttgtacgacgagtgctgcaaatttgtgcagtgatagtgcagctttgcgcaactttaaaaggcagtattttgaacaccg
                                                                                         270
catgcgatgtatagcgcctcgaatagccatttgtgcaataaaagcaaatcttttcattgcaactcatatcaaatttttttaagttaaagag
                                                                                         360
aatatcgtcgatccatagttgagaaaaataagctagaatgatggccggtttagaaagaattgcgtcaggattaccctggcatcattcgtg
         -35                     -10                                                     450
gagaaaaaaatgagcttatctgaaagtttagctaaatacggcattactggtgctaccaacatcgtccacaatccttctcacgaggagttg
 SD        M  S  L  S  E  S  L  A  K  Y  G  I  T  G  A  T  N  I  V  H  N  P  S  H  E  E  L
                                                                                         540
tttgctgctgagactcaggcttctttagaaggcttcgagaagggcactgtaaccgagatgggtgcagtaaacgttatgactggcgtttac
 F  A  A  E  T  Q  A  S  L  E  G  F  E  K  G  T  V  T  E  M  G  A  V  N  V  M  T  G  V  Y
                                                                                         630
accggccgttcccctaaggacaagttcattgttaagaacgaggcctccaaggagatctggtggacttctgatgagttcaagaacgacaac
 T  G  R  S  P  K  D  K  F  I  V  K  N  E  A  S  K  E  I  W  W  T  S  D  E  F  K  N  D  N
                                                                                         720
aagccagttaccgaagaggcatgggctcagttaaaggctttagcaggcaaggagctctctaacaagcctttatacgttgttgatctcttc
 K  P  V  T  E  E  A  W  A  Q  L  K  A  L  A  G  K  E  L  S  N  K  P  L  Y  V  V  D  L  F
                                                                                         810
tgtggtgctaacgagaacacccgtctgaagatccgtttcgttatggaagtagcttggcaggctcacttcgtaaccaacatgttcatccgt
 C  G  A  N  E  N  T  R  L  K  I  R  F  V  M  E  V  A  W  Q  A  H  F  V  T  N  M  F  I  R
                                                                                         900
cctactgaggaagagttaaagggcttcgagccagacttcgtagttctgaacgcttcaaaggctaaggttgagaacttcaaggagttaggc
 P  T  E  E  E  L  K  G  F  E  P  D  F  V  V  L  N  A  S  K  A  K  V  E  N  F  K  E  L  G
                                                                                         990
ctcaactctgagactgctgttgtattcaacctcgcagagaagatgcagatcatcctcaacacctggtacggtggtgagatgaagaagggc
 L  N  S  E  T  A  V  V  F  N  L  A  E  K  M  Q  I  I  L  N  T  W  Y  G  G  E  M  K  K  G
                                                                                        1080
atgttctccatgatgaacttctacttaccattacagggcatcgctgcaatgcactgctctgctaacaccgacctcgagggcaagaacact
 M  F  S  M  M  N  F  Y  L  P  L  Q  G  I  A  A  M  H  C  S  A  N  T  D  L  E  G  K  N  T
                                                                                        1170
gctatcttcttcggtctgtcaggcactggtaagaccaccttatctaccgatcctaagcgtttactcatcggtgacgacgagcacggttgg
 A  I  F  F  G  L  S  G  T  G  K  T  T  L  S  T  D  P  K  R  L  L  I  G  D  D  E  H  G  W
                                                                                        1260
gacgatgatggcgtattcaacttcgaaggcggctgctacgctaaggttattaacctctctaaggaaaacgagccagacatctggggcgct
 D  D  D  G  V  F  N  F  E  G  G  C  Y  A  K  V  I  N  L  S  K  E  N  E  P  D  I  W  G  A
                                                                                        1350
atcaagcgtaacgctttattagagaacgtaactgttgatgctaacggcaaggttgactttgcagacaagtctgtaactgagaacacccgt
 I  K  R  N  A  L  L  E  N  V  T  V  D  A  N  G  K  V  D  F  A  D  K  S  V  T  E  N  T  R
                                                                                        1440
gtttcttacccaatcttccacattaaaaacatcgttaagccagtttctaaggctccagctgctaagcgcgtaatcttcttatctgctgat
 V  S  Y  P  I  F  H  I  K  N  I  V  K  P  V  S  K  A  P  A  A  K  R  V  I  F  L  S  A  D
                                                                                        1530
gctttcggtgtattacctccagtatctatcctttctaaggagcagactaagtactacttcctctctggcttcaccgctaagttagctggt
 A  F  G  V  L  P  P  V  S  I  L  S  K  E  Q  T  K  Y  Y  F  L  S  G  F  T  A  K  L  A  G
                                                                                        1620
accgagcgtggcattaccgagcctaccccaaccttctcttcatgcttcggtgctgctttcttaaccttacctccaaccaagtacgctgaa
 T  E  R  G  I  T  E  P  T  P  T  F  S  S  C  F  G  A  A  F  L  T  L  P  P  T  K  Y  A  E
                                                                                        1710
gttctggttaagagaatggaagcttccggcgctaaggcttacttagtaaacactggctggaacggcactggcaagcgtatctccattaag
 V  L  V  K  R  M  E  A  S  G  A  K  A  Y  L  V  N  T  G  W  N  G  T  G  K  R  I  S  I  K
                                                                                        1800
gatacccgtggcattatcgacgcaatcttagacggttctatcgatactgcaaacaccgcaaccatcccttacttcaacttcaccgttcct
 D  T  R  G  I  I  D  A  I  L  D  G  S  I  D  T  A  N  T  A  T  I  P  Y  P  N  P  T  V  P
                                                                                        1890
actgagttaaagggcgttgacaccaagattctggacccacgtaacacctacgctgacgcttcagagtgggaagtaaaggctaaggactta
 T  E  L  K  G  V  D  T  K  I  L  D  P  A  N  T  Y  A  D  A  S  E  W  E  V  K  A  K  D  L
                                                                                        1980
gctgagcgcttccagaagaacttcaagaagttcgagtctttaggtggtgacttagttaaggctggtccacagttataattcctgtctgac
 A  E  R  F  Q  K  N  F  K  K  F  E  S  L  G  G  D  L  V  K  A  G  P  Q  L ***
                                                                                        2070
cctttaagggcagaatagatgaattactaacgggcccgaggtgctatatgcgcctcgggccttttgtatttgtatagacaaggaagtcta
                                                                                        2160
taggaggacaatttggcaaagaagccttacattacccctcaggatatgagttaaaaaggaattagattatctttggcttgaaaaacgtcc
                                                                                        2250
agatgtaactgctaaggtttcatgggcctgcaagcttaggagatcgctccgagaacgctgactatcagtacaacaagcgtttgcttgctc
                                         2295
agattgaccgtcgcattcgctatctacgtaagattcttaatgatc
```

FIG. 2. Nucleotide and deduced amino acid sequences of *A. succiniciproducens pckA*. The putative Shine-Dalgarno (SD) and amino acid sequences determined by N-terminal amino acid sequencing are underlined. The stop codon is indicated by three asterisks. ⇓ indicates the *Sac*I site used to join *pckA*'s 5′ and 3′ parts. Detected transcription start sites are denoted by ↓. Putative promoter sequences are denoted by −10 and −35 and are in italics. Horizontal arrows indicate a terminator-like, inverted repeat sequence.

TABLE 1. Comparison of the codon frequency in *A. succiniciproducens* and *E. coli pckA* genes and the average codon frequency in *E. coli* genes

Amino acid	Codon	Codon frequency in *pckA* gene		Avg codon frequency in *E. coli* genes[b]
		A. succiniciproducens	*E. coli*[a]	
Phe	TTT	0.4	1.7	1.3
	TTC	5.6	3.7	2.2
Leu	TTA	4.9	0.2	0.2
	TTG	0.2	0.7	0.7
	CTT	0.2	0.6	0.6
	CTC	1.7	1.3	1.3
	CTA	0.0	0.0	0.0
	CTG	0.9	5.6	5.6
Ile	ATT	1.5	0.9	1.3
	ATC	3.8	3.0	3.5
	ATA	0.0	0.4	0.0
Met	ATG	2.3	2.2	2.0
Val	GTT	3.9	2.4	2.4
	GTC	0.2	1.1	1.1
	GTA	2.6	0.6	0.6
	GTG	0.0	2.0	2.0
Ser	TCT	3.6	1.3	1.3
	TCC	0.9	1.3	1.5
	TCA	0.8	0.4	0.4
	TCG	0.0	0.7	0.6
	AGT	0.2	0.2	0.2
	AGC	0.2	0.6	0.6
Pro	CCT	1.9	0.4	0.5
	CCC	0.0	0.0	0.3
	CCA	2.3	0.9	0.7
	CCG	0.0	3.0	2.5
Thr	ACT	3.0	2.6	1.1
	ACC	4.7	3.7	2.4
	ACA	0.0	0.4	0.3
	ACG	0.0	0.2	0.8
Ala	GCT	7.1	2.2	2.6
	GCC	0.2	1.5	2.2
	GCA	1.7	1.3	2.3
	GCG	0.0	3.2	3.2

Amino acid	Codon	Codon frequency in *pckA* gene		Avg codon frequency in *E. coli* genes[b]
		A. succiniciproducens	*E. coli*[a]	
Tyr	TAT	0.0	1.7	1.0
	TAC	2.4	1.5	1.5
His	CAT	0.0	1.3	0.7
	CAC	1.1	1.3	1.2
Gln	CAA	0.0	0.6	1.0
	CAG	1.5	2.2	3.2
Asn	AAT	0.2	0.4	1.0
	AAC	5.8	4.1	2.8
Lys	AAA	0.4	4.5	4.1
	AAG	8.5	1.5	1.3
Asp	GAT	1.7	3.7	2.5
	GAC	3.2	2.6	3.0
Glu	GAA	1.9	1.3	4.9
	GAG	5.8	3.7	1.8
Cys	TGT	0.2	0.2	0.4
	TGC	0.6	0.6	0.5
Trp	TGG	1.7	1.7	0.7
Arg	CGT	2.1	2.2	3.1
	CGC	0.4	1.7	2.0
	CGA	0.0	0.9	0.2
	CGG	0.0	0.2	0.2
	AGA	0.2	0.0	0.06
	AGG	0.0	0.6	0.04
Gly	GGT	3.0	3.0	3.8
	GGC	4.7	4.9	3.1
	GGA	0.0	0.4	0.4
	GGG	0.0	0.9	0.6

[a] *E. coli pckA* corrected sequence (see Materials and Methods).
[b] Codon frequency in *E. coli* genes was calculated from reference 1.

ciproducens pckA leucine codons are TTA, whereas 67% are CTG in *E. coli* genes. *A. succiniciproducens pckA* alanine codons are GCT in 79% of the cases, whereas there is almost no preference for any Ala codon in *E. coli* genes. Almost all *A. succiniciproducens pckA* lysine codons are AAG (96%); 76% are AAA in *E. coli* genes. Finally, 76% of *A. succiniciproducens pckA* glutamate codons are GAG, versus 73% GAA codons in *E. coli* genes. Since *pckA* is the first *A. succiniciproducens* gene to be cloned and sequenced, it is too early to say if the different codon usage for leucine, alanine, lysine, and glutamate residues corresponds to a general trend in the *A. succiniciproducens* genome.

Determination of the transcription start site. To identify the *A. succiniciproducens* promoter, the transcriptional start site was determined by primer extension. The oligonucleotide primer was complementary to nt 30 to 52 in the coding se-

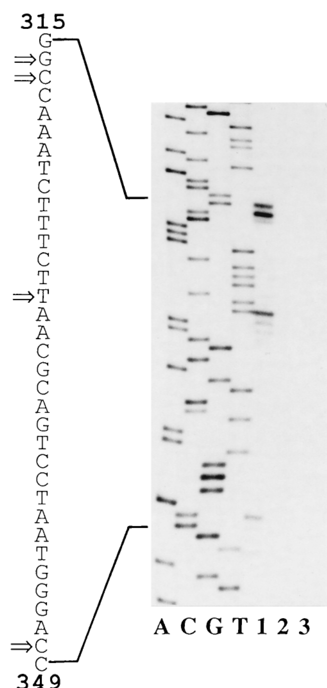

FIG. 3. Determination of *A. succiniciproducens pckA* transcription start site by primer extension. Arrows indicate the sequence positions of the major extension products' start sites. Lanes: A, C, G, and T, control sequencing reactions with the primer used for primer extension; 1, primer extension products; 2, control (no RNA added); 3, control (no primer added).

quence. Template RNA was extracted from an *A. succiniciproducens* culture in the late exponential growth phase. Four major extension products were observed (Fig. 3). The most abundant product ended at C_{317} on the coding strand (Fig. 3). Additional extension products ending at positions G_{316}, T_{329}, and C_{348} were observed. Since no reaction products extended farther than G_{316}, G_{316} probably represents the earliest *pckA* transcription start point in *A. succiniciproducens.*

A putative −10 promoter region ($T_{304}AGAAT_{309}$), highly similar to the *E. coli* −10 promoter consensus, was identified 8 bp upstream from C_{317}. This −10 region is preceded by the sequence $T_{282}CCATA_{287}$, reminiscent of the *E. coli* −35 promoter consensus region. The potential −10 and −35 regions are separated by a typical 16-nt interregion spacing. Interestingly, a sequence ($T_{289}TGAGA_{294}$) almost identical to the *E. coli* −35 consensus region is present 11 nt upstream of the −10 region. At least 20% of the 112 *E. coli* promoters aligned by Harley and Reynolds (11) contain a sequence (7 to 12 nt upstream of the −10 region) that is more closely related to the −35 consensus than is the sequence considered to be the functional −35 region. The significance of these additional consensus sequences for transcription initiation is unknown. Whether or not the putative promoter identified is the functional promoter in *A. succiniciproducens* has to be proven. Little is known about *A. succiniciproducens*'s taxonomic position, and *pckA* is the first *A. succiniciproducens* gene for which a promoter study has been performed. Thus, it is unknown if *A. succiniciproducens* promoter sequences are expected to highly resemble *E. coli* promoters.

Comparison of protein sequences. *A. succiniciproducens pckA* is the 11th ATP/ADP-dependent PCK gene to be sequenced entirely. A protein alignment was built that included these 11 sequences and 2 truncated ones (not shown). PckA sequences showed a high level of conservation (58 to 80% similarity and 39 to 70% identity). The *A. succiniciproducens* PCK was more closely related to ATP/ADP-dependent PCKs from gram-negative bacteria (79 to 80% similar and 67 to 70% identical to the *E. coli*, *S. typhimurium*, and *H. influenzae* PCKs) (Fig. 4) than to gram-positive bacteria. *A. succiniciproducens* PCK contained all of the functional residues (i.e., ATP, Mg^{2+}, and PEP or OAA binding sites) (Table 2) found in the *E. coli* PCK structure and conserved in all ATP/ADP-dependent PCKs (17, 29). Only four residues conserved in all other ATP/ADP-dependent PCKs are substituted in *A. succiniciproducens* PCK. *A. succiniciproducens* PCK's Asn_{197}, Asp_{229}, Ile_{367}, and Pro_{411} replace Gly, Gly, Arg or Lys, and His, respectively, in the other enzymes. The significance of these substitutions is unknown. With its high level of identity to the *E. coli* PCK and the presence of only three one- or two-residue gaps (all located in loops) in its alignment to the *E. coli* PCK, *A. succiniciproducens* PCK probably adopts a three-dimensional structure almost completely superimposable to the *E. coli* PCK's.

Gene expression and recombinant enzyme purification. Since we were not able to join both of *A. succiniciproducens pckA*'s parts in pUC18, we subcloned them into the *Ehe*I-*Sal*I sites of the expression vector pProEX-1, producing plasmid pProPC1. The *pckA* 5′ end was amplified from pPCK2 with primer 3 and the M13 reverse primer. The PCR product was digested with *Nsi*I. The product was then treated with mung bean nuclease (to become compatible with the vector's *Ehe*I site), digested with *Eco*RI, and cloned into pProEX-1's *Ehe*I-*Eco*RI sites. The *pckA* 3′ end was then cloned in the *Sac*I-*Sal*I sites of the preceding construct. Upon induction with 0.6 mM isopropyl-β-D-thiogalactopyranoside (IPTG), *E. coli* DH5α

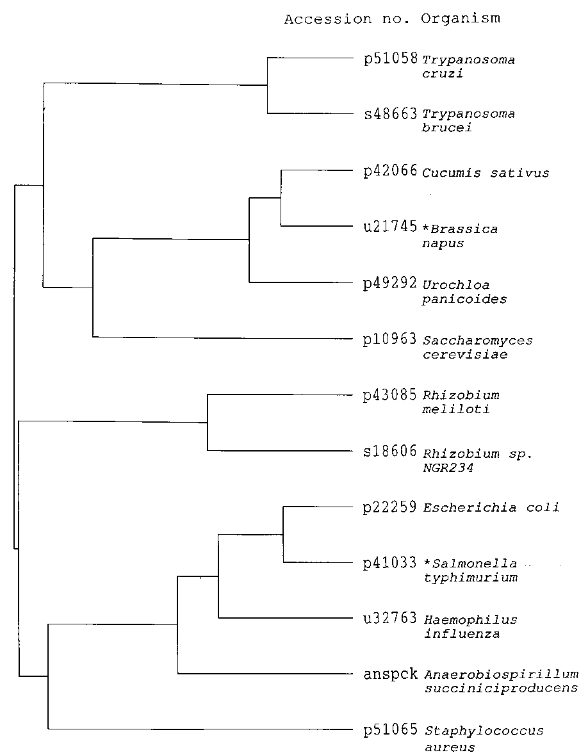

FIG. 4. Dendrogram of the known ATP/ADP-dependent PCKs. PCK sequences were obtained from GenBank (U21745 and U32763), SwissProt (P10963, P22259, P41033, P42066, P43085, P49292, P51058, and P51065) and pir2 (S18606 and S48663). *, truncated sequences.

TABLE 2. Conservation of PCK active site residues (17, 29) in *A. succiniciproducens* PCK

Binding site	Motif or residue	Characteristic(s)
ATP		
Phosphate binding	His_{232} (*E. coli*) His_{225} (*A. succiniciproducens*)	*E. coli* His_{232} interaction with the ATP γ-phosphate
Phosphate and Mg^{2+} binding (kinase 1a motif)	G XXGXGKT (consensus) $G_{248}LSGTGKT_{255}$ (*E. coli*) $G_{242}LSGTGKT_{249}$ (*A. succiniciproducens*)	*E. coli* S_{250}, K_{254}, and T_{255} interaction with ATP and coordinate Mg^{2+}
Mg^{2+} binding (kinase 2 motif)	X XXXD (consensus) $L_{265}IGDD_{269}$ (*E. coli*) $L_{259}IGDD_{263}$) (*A. succiniciproducens*)	*E. coli* D_{269} interaction with Mg^{2+} through a water molecule
Ribose binding	K XXXLXXXXE (consensus) $K_{288}TIKLSKEAE_{297}$ (*E. coli*) $K_{282}VINLSKENE_{291}$ (*A. succiniciproducens*)	Direct and water-mediated interactions with ATP ribose
Adenine binding	R XXXXXT (consensus) $R_{449}ISIKDT_{455}$ (*E. coli*) $R_{443}ISIKDT_{449}$ (*A. succiniciproducens*)	*E. coli* R_{449}, I_{452}, and T_{455} interaction with ATP adenine
PEP or OAA	R_{65}, Y_{207}, K_{213}, and R_{333} (*E. coli*) R_{60}, Y_{200}, K_{206}, and R_{327} (*A. succiniciproducens*)	Interaction with oxalate in *E. coli* PCK-ATP-Mg^{2+}-oxalate crystal structure; *E. coli* R_{333} creation of bridge between ATP γ-phosphate and oxalate

(pProPC1) overexpressed the recombinant protein, which represented approximately 20% of the total cell protein (according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] gel scanning data). Since the protein expressed in pProPC-1 is a fusion protein containing an N-terminal His tag, we used a Ni-NTA resin to purify the recombinant enzyme by affinity chromatography. Purification with the Tris-HCl buffer-based protocol yielded homogeneous recombinant enzyme in a quantitative yield (Fig. 5). With 22 extra N-terminal amino acids, the recombinant PCK was perfectly active, with a specific activity of 73.1 U/mg protein. After TEV protease treatment—followed by a second Ni-NTA affinity chromatography to remove the cut His tag and the uncut protein fractions—the recombinant enzyme had the same specific activity (92 U/mg) as the native PCK (91 U/mg) and could be detected as a single band by SDS-PAGE. It had the same molecular mass as the native enzyme (Fig. 5).

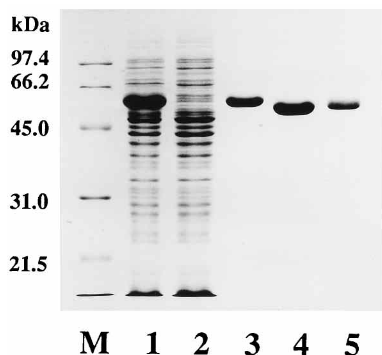

FIG. 5. SDS-PAGE of *E. coli* DH5α(pProPC-1) crude cell extract and purified recombinant PEP carboxykinase. Lanes: M, molecular mass standards; 1, *E. coli* DH5α(pProPC-1) crude cell extract; 2, unbound proteins after Ni-NTA affinity chromatography; 3, purified recombinant fusion protein His tag-PEP carboxykinase; 4, TEV protease-treated recombinant PEP carboxykinase; 5, purified native *A. succiniciproducens* PEP carboxykinase.

Kinetic properties. The *A. succiniciproducens* PCK kinetic parameters were determined in the presence of both $MnCl_2$ (5 mM) and $MgCl_2$ (10 mM) (Table 3). The apparent discrepancy observed among the three V_{max} values obtained for the carboxylation reaction is due to the conditions in which the K_ms for PEP and ADP were determined. These K_ms were determined in the presence of only 100 mM $NaHCO_3$, which was rate limiting. Higher $NaHCO_3$ concentrations induced the formation of too many bubbles in the reaction cuvette. The higher V_{max} (119 U/mg) obtained while determining the enzyme's K_m for HCO_3^- represents the correct V_{max} for the carboxylation reaction. Since Mn^{2+} and Mg^{2+} have a synergistic activating effect on enzyme activity (24), we wanted to study the effect Mn^{2+} had on enzyme kinetic properties. In the carboxylation direction, K_ms for PEP and ADP are similar to the K_ms for PEP (0.54 mM) and for ADP (0.42 mM) determined in the presence of Mg^{2+} only (24). The enzyme's affinity for HCO_3^-, however, decreased twofold in the presence of Mn^{2+}, with its K_m for HCO_3^- going from 17 mM in the presence of Mg^{2+}

TABLE 3. Comparison of *A. succiniciproducens* PCK's kinetic parameters with the kinetic parameters of other ATP/ADP-dependent PCKs

Substrate	Apparent K_m [mM] (apparent V_{max} [U/mg of protein]) of PCK from[a]:			
	A. succiniciproducens	*E. coli*	*S. cerevisiae*	*T. cruzi*
Carboxylation				
PEP	0.46 ± 0.05 (82.8 ± 2.5)	0.07	ND[b]	0.035 (3.22)
ADP	0.44 ± 0.16 (82.8 ± 8.2)	0.05	ND	0.017 (3.09)
$NaHCO_3$	30 ± 6.4 (119.0 ± 10.1)	13.0	ND	2.77 (3.40)
Decarboxylation				
OAA	0.25 ± 0.11 (20.2 ± 3.5)	0.67	0.46	0.044 (28)
ATP	0.032 ± 0.012 (28.8 ± 0.62)	0.06	0.05	0.027 (32)

[a] Data are from reference 15 for *E. coli*, reference 12 for *S. cerevisiae*, and reference 6 for *T. cruzi*.
[b] ND, not determined.

only to 30 mM in the presence of Mn^{2+}. While the enzyme's K_ms for its substrates PEP, ADP, and HCO_3^- remained marginally affected by Mn^{2+}, its V_{max} (10 U/mg in the presence of Mg^{2+} only) (24) increased 12 times upon addition of Mn^{2+}. Using the enzyme's K_m for HCO_3^- (the substrate with the highest K_m)—and a V_{max} of 119 U/mg in the presence of Mn^{2+}—values for the V_{max}/K_m ratio were 0.58 and 4.0 in the absence and presence of Mn^{2+}, respectively, showing that Mn^{2+} increased the enzyme catalytic efficiency almost seven times.

In the decarboxylation reaction, the presence of added Mn^{2+} strongly affected the enzyme's affinity for its substrates. Initially 1.2 and 2.3 mM, respectively, in the presence of Mg^{2+} only, K_ms for OAA and ATP dropped 9 and 72 times, respectively, upon addition of Mn^{2+} to the assay. With the enzyme's K_m for OAA and an averaged V_{max} of 24 U/mg, the V_{max}/K_m ratio in the presence of Mn^{2+} was 96, showing that *A. succiniciproducens* PCK's catalytic efficiency is 24 times higher for decarboxylation than for CO_2 fixation.

DISCUSSION

The *A. succiniciproducens pckA* gene was cloned by two approaches. (i) The 3′ end was cloned by hybridization of an *A. succiniciproducens* genomic library in *E. coli* with a PCR-generated homologous probe. (ii) The 5′ end was cloned by ligation-mediated PCR. The complete *pckA* gene was successfully expressed in *E. coli*, and the recombinant enzyme was purified with a powerful purification procedure. The *A. succiniciproducens* PCK sequence appeared to be typical of an ATP-dependent PCK.

The obligate anaerobe *A. succiniciproducens* produces industrial-level concentrations of succinic acid—up to 35 g/liter. Under conditions favoring succinate production (pH 6.2 and high CO_2 concentration), 65% of the glucose carbon used by *A. succiniciproducens* is directed toward succinate production (from PEP, by CO_2 fixation) (26). Under these conditions, PCK expression increases significantly (it represents up to 10% of whole-cell protein), and high PCK levels can be detected. This physiological evidence demonstrates that when conditions favor succinate production, *A. succiniciproducens* PCK, a key enzyme in succinate production, fixes CO_2 to form OAA. The *A. succiniciproducens pckA* gene was cloned and sequenced, and its product was compared to those of other ATP/ADP-dependent PCKs. ATP/ADP-dependent PCKs physiological function can either be CO_2 fixation—leading to succinate production—or decarboxylation. PCK has been shown to be the CO_2-fixing enzyme in trypanosomatids (6). In the facultative anaerobe *Actinobacillus* sp., production of succinate as a major fermentation product is also related to high PCK activity (31). In *Haemophilus* species (known to ferment glucose into acetate, lactate, and succinate and belonging to the same *Pasteurellaceae* family as *Actinobacillus*), PCK is probably involved in succinate production. On the other hand, rhizobia are nonfermentative, aerobic bacteria, and the *E. coli* PCK seems only marginally, if at all, involved in succinate production (31) from glucose. The dendrogram (Fig. 4) illustrating the similarity scores between ATP/ADP-dependent PCKs seems to respect the phylogenetic relationships among organisms. These similarity scores, ignorant of the enzyme's physiological functions, suggest that no specific structural features can be found in CO_2-fixing ATP/ADP-dependent PCKs.

The *pckA* transcription start site was determined by primer extension. The most abundant extension product ended at C_{318}, which allowed us to identify putative −10 and −35 promoter regions. No products extending farther than G_{317} were detected (G_{317} represents the earliest *pckA* transcription starting point in cells). To determine whether transcripts starting at T_{330} and C_{349} exist in the cells (and are not reverse transcriptase pausing points), nuclease S1 mapping should be used.

To obtain enough pure protein for structural and kinetic investigations, we chose to purify the recombinant protein by affinity chromatography. Expression of *A. succiniciproducens pckA*, cloned in vector pProEX-1 and expressed in *E. coli* DH5α, resulted in high levels of expression (approximately 20% of total cell protein). The purified recombinant protein, devoid of its N-terminal His tag but still supposed to contain one additional glycine at its N terminus, was indiscernible from the native protein by SDS-PAGE and in activity assays. Since the presence of an extra glycine at the N terminus is not expected to alter the protein folding, this affinity chromatography purification technique, which allows the selective purification of the *A. succiniciproducens* enzyme without contamination by the *E. coli* enzyme, will be used to purify large amounts of PCK for future structure-function studies.

The *A. succiniciproducens* PCK affinity constants listed in Table 1 were compared to those determined for other ATP/ADP-dependent PCKs. The *A. succiniciproducens* PCK K_ms for PEP and ADP are 1 order of magnitude higher than the corresponding constants for the *E. coli* and *Trypanosoma cruzi* enzymes. These differences in K_m values for PEP and ADP might not affect these enzymes' activity, since each of these three enzymes has a K_m for HCO_3^- 2 orders of magnitude higher than its K_ms for PEP and ADP. *A. succiniciproducens* PCK K_m values for OAA and ATP are similar to the *E. coli* and *S. cerevisiae* ones, but they are 1 order of magnitude higher than the *T. cruzi* K_ms. The *T. cruzi* PCK is the only PCK for which V_{max} values are available (Table 3). For the *E. coli* enzyme, the carboxylation reaction rate was determined by three different methods. The reaction rates were 29.5, 28.3, and 20.7 U/mg of enzyme. If we consider these rates to represent approximately the *E. coli* enzyme V_{max}, *A. succiniciproducens* PCK appears to have a V_{max} (119 U/mg) for CO_2 fixation about 4.5- and 37-times higher, respectively, than those of the *E. coli* and *T. cruzi* PCKs.

The kinetic properties compared in Table 3 were not determined under similar experimental conditions. *T. cruzi* K_ms for ATP and ADP, for instance, were determined under conditions optimized for PCK activity (presence of various concentrations of Mn^{2+}, always 0.5 mM superior to the ATP/ADP concentration) (6). Other enzymes' K_ms for ADP and ATP were determined under nonoptimized conditions, in the presence of fixed Mn^{2+} and/or Mg^{2+} (references 12 and 15 and this study). The comparisons based on the Table 3 data should therefore be considered cautiously. Still, *A. succiniciproducens* PCK seems to be a significantly better CO_2-fixing enzyme than the *E. coli* and *T. cruzi* enzymes: it has significantly higher V_{max} and catalytic efficiency (represented by a V_{max}/K_m ratio of 4.0) in the carboxylation reaction than those of the *E. coli* and *T. cruzi* PCKs (their V_{max}/K_m ratios are 2.0 and 1.2, respectively, as calculated from Table 3). We already knew that metabolic regulation was essential in determining *A. succiniciproducens* PCK's function as a CO_2-fixing enzyme (24). In addition, the *A. succiniciproducens* PCK enzyme itself appears to be optimally designed for its function.

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