Characterization of acetyl-CoA and propionyl-CoA carboxylases encoded by *Leptospira interrogans* serovar Lai: an initial biochemical study for leptospiral gluconeogenesis via anaplerotic CO₂ assimilation

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*Leptospira interrogans* is the causative agent of leptospirosis. The *in vitro* growth of *L. interrogans* requires CO₂ and a partial 3-hydroxypropionate pathway involving two acyl-CoA carboxylases was suggested by genomic analysis to assimilate CO₂. Either set of the candidate genes heterologously co-expressed in *Escherichia coli* was able to demonstrate both acetyl-CoA carboxylase (ACC) and propionyl-CoA carboxylase (PCC) activities. The trisubunit holoenzyme (LA_2736-LA_2735 and LA_3803), although failed to be purified, was designated ACC based on its substrate preference toward acetyl-CoA. The partially purified bi-subunit holoenzyme (LA_2432-LA_2433) has a considerably higher activity against propionyl-CoA as the substrate than that of acetyl-CoA, and thus, designated PCC. Native polyacrylamide gel electrophoresis indicated that this PCC has a molecular mass of around 669 kDa, suggesting an α₄β₄ γ-quaternary structure and both structural homology modeling and site-directed mutagenesis analysis of its carboxyltransferase subunit (LA_2433) indicated that the A431 residue located at the bottom of the putative substrate binding pocket may play an important role in substrate specificity determination. Both transcriptomic and proteomic data indicated that enzymes involved in the suggested partial 3-hydroxypropionate pathway were expressed *in vivo* in addition to ACC/PCC and the homologous genes in genomes of other *Leptospira* species were re-annotated accordingly. However, as the in vitro detected specific activity of ACC in the crude cell extract was too low to account for the growth of the bacterium in Ellinghausen–McCullough–Johnson–Harris minimal medium, further systematic analysis is required to unveil the mechanism of gluconeogenesis via anaplerotic CO₂ assimilation in *Leptospira* species.

**Keywords** acetyl-CoA carboxylase; propionyl-CoA carboxylase; *Leptospira interrogans*; CO₂ assimilation

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**Introduction**

*Leptospira interrogans* is the causative agent of the zoonotic leptospirosis [1,2]. Basic studies related to the biology of *Leptospira* have mainly focused on potential virulence factors such as lipopolysaccharide [3–7], dynamics of motility [8–12], and outer-membrane proteins [13–20]. However, the study of its metabolism has been largely limited to those special or unique for the spirochetes, such as the biosynthesis of branch-chain amino acids [21–23] and the metabolism of lipids [20,24,25].

Although the activities of both key enzymes of the glyoxylate pathway, isocitrate lyase, and malate synthase were detected in *Leptospira biflexa* [26], neither of them
were encoded in any genomes of the pathogenic *L. interrogans* [27] or of the saprophytic *L. biflexa* [28]. On the other hand, CO₂ was shown to be essential for the *in vitro* growth of *L. interrogans* [29, 30] and the *L. interrogans* serovar Lai genome was annotated to encode putative subunits of two potential acyl-CoA carboxylases [31–35] by five genes (*LA_2432, LA_2433, LA_2736, LA_2735,* and *LA_3803*), which inferred a partial 3-hydroxypropionate pathway for anaplerotic CO₂ assimilation [27]. In this study, we experimentally characterized these two acyl-CoA carboxylases and found that *LA_2432* and *LA_2433* constituted a propionyl-CoA carboxylase (PCC), whereas *LA_2736*, *LA_2735*, and *LA_3803* composed an acetyl-CoA carboxylase (ACC). The apparently conserved substrate binding pocket of carboxytransferase subunit in the leptospiral PCC was predicted by structure homology modeling, and was further proved by site-directed mutagenesis. By sequence similarity comparison, the homologous genes encoded by other leptospires were re-annotated based on the enzymatic characterization. In addition to ACC and PCC, transcriptomic and proteomic data indicated that other enzymes should be involved in the suggested partial 3-hydroxypropionate pathway expressed *in vivo*, and we noticed that future systematic analysis was required to unveil all the possible mechanisms related to gluconeogenesis via anaplerotic CO₂ assimilation in *Leptospira* species.

**Materials and Methods**

**Bacteria strains and plasmids**

Bacterial strains and plasmids used in this study were summarized in **Supplementary Table 1** with their related reference information. The virulent strain 56601 of *L. interrogans* serogroup icterohaemorrhagiae serovar Lai was grown in Ellinghausen–McCullough–Johnson–Harris (EMJH) liquid medium at 28°C [36, 37], and the cells were harvested at their exponential growth phases. Competent cells of *Escherichia coli* DH5α and BL21 (DE3) used for subcloning and transformation, respectively, were prepared according to the literature [38]. The growth condition of *E. coli* BL21 (DE3) harboring the expression vectors for recombinant protein expression will be described below.

**Reverse transcription-polymerase chain reaction**

Total RNA was extracted from the *in vitro* cultured *L. interrogans* cells using the Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. RNA was treated with DNase I (Promega, Madison, USA) for 30 min at 37°C to eliminate DNA contamination. cDNA was synthesized from 2 μg RNA using the Superscript III reverse transcriptase (Invitrogen) with 6-bp random primers in a 20-μl volume. Targeted gene expression level was analyzed by semi-quantitative polymerase chain reaction (PCR) (MyCycler; Bio-Rad, Hercules, USA) using 20-ng cDNA as the template. The same amount of RNA was used for negative control, genomic DNA as positive control, and the primers were shown in **Supplementary Table 2**. The PCR conditions are shown as follows: 95°C, 8 min, followed by 35 amplification cycles of 95°C, 30 s; 56°C, 30 s; and 72°C, 40 s; last one cycle of 72°C, 10 min.

**DNA manipulation**

Genomic DNA was extracted using genomic DNA isolating kit (Shanghai Watson Biotech Co. Ltd, Shanghai, China). The PCR primers were listed in **Supplementary Table 3**. PCR reaction mixture (50 μl) contained 100-ng genomic DNA, 0.4 μM of each primer, 0.25 μM dNTP (TaKaRa, Dalian, China), 5 μl 10 × *Pfu* buffer (Mg²⁺), and *Pfu* DNA polymerase (Fermentas International Inc., Burlington, Canada), or 10 μl 5 × Fast*Pfu* buffer (Mg²⁺) and 2.5 U TransStart Fast*Pfu* DNA polymerase (Beijing TransGen Biotech Co., Ltd, Beijing, China). PCR products were purified by Gel extraction kit (Shanghai Watson Biotech Co. Ltd), and then accP, accB, accC, pccC, and pccC genes were cloned into pRSFduet-1 vector (Merck, San Diego, USA).

**Protein expression, purification, and analysis**

Homologous amino acid sequences were aligned using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Conserved protein structural domains and motifs were searched in NCBI Conserved Domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

The *E. coli* BL21 (DE3) harboring the expression vector was grown in Luria Bertani medium containing 50 μg/ml kanamycin at 37°C on a rotary shaker at 200 rpm. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (1 mM) to bacterial culture for 20 h at 16°C until OD₆₀₀ reached 0.6–0.8. Then the cells were harvested, resuspended in buffer A (50 mM Tris–HCl, pH 8.0, 300 mM NaCl, 0.75 mM DTT, 1 mM EDTA, and 10% glycerol) [39], and disrupted by sonication with ultrasonic cell crusher (Scientz Biotechnology Co., Ltd, Ningbo, China) set at 200 W for 5-s pulse on, 15-s pulse off for 30 times. After being spun on the CR21GII centrifuge (Hitachi, Tokyo, Japan) for 30 min at 20,000 g at 4°C, the supernatant was retained. The protein concentration was determined by the Braford method [40].

The recombinant protein purified with affinity chromatography (Ni-NTA Superflow; Qiagen, Hilden, Germany), washed with buffer A supplemented with 20, 40 mM imidazole, and eluted with 250 mM imidazole. The eluted proteins were dialyzed against 100 mM potassium phosphate...
buffer (pH 7.6, with 0.75 mM DTT, 1 mM EDTA, and 20% glycerol) through the 10 kDa molecular cut-off dialysis membrane (Union Carbride, South Houston, USA) at 4°C. The buffer was changed three times, i.e., 1 h each, for twice, and then overnight. After that, the proteins were concentrated by ultrafiltration (Amicon, Danvers, USA) and stored at −80°C. The protein purity was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% polyacrylamide for the separating gel and 5% for the stacking gel) with Bio-Rad mini-gel apparatus. Target protein bands in SDS-PAGE are quantified using bovine serum albumin (BSA) as standard protein and analyzed by Gel-Pro analyzer 4 (MediaCybernetics, Bethesda, USA). Native-PAGE was carried out at 4°C using 6% polyacrylamide for 4 h, stained with Coomassie brilliant blue R-250. The native marker (HMW calibration kit for native electrophoresis) was purchased from Amersham (Buckinghamshire, UK), and the protein bands were identified by the Shanghai Applied Protein Technology Co., Ltd (Shanghai, China).

Enzyme assays
The enzyme assay was measured by the incorporation of H14CO3- into acid-stable reaction products. The ACC and PCC [39,41–43] reaction mixture contained 100 mM potassium phosphate, pH 8.0, 3 μg/μl of BSA, 10 mM MgCl2, 2.4 mM ATP, 10 mM KCl, 9.6 mM NaH14CO3 [specific activity, 84 mCi/mmol (310.8 MBq/mmol)], different concentrations of substrate [acetyl-CoA or propionyl-CoA, HPLC pure (95%), from Sigma, St Louis, USA], and cell-free protein extract or partially purified protein in a total reaction volume of 100 μl. The reaction was initiated by adding NaH14CO3, allowed to proceed at 30°C for 15 min, and stopped with 200 μl of 6 M of HCl. The contents of the tubes were then evaporated to dryness at 95°C. The residue was resuspended in 100 μl of water, and then 1 ml of Ultima gold liquid scintillation medium (PerkinElmer, Waltham, USA) was added. The 14C radioactivity was determined in a multi-purpose scintillation counter (Beckman, Pasadena, USA). All kinetic data were analyzed using Prism 4.0 for Windows (GraphPad).

3D Structure simulation
The homology model of L. interrogans PccB was built using the homology modeling module of MOE 2009.10 with the ligand supported option. The template was the crystal structure of PCC from Streptomyces coelicolor complexed with propionyl-CoA (PDB ID: 1XNY), which provides detailed binding information of propionyl-CoA with PccB. First, the sequence alignment was performed using the BLOSUM62 score matrix of MOE with default settings, which resulted in 34% homology identity. Ten possible models were built using the default methods for homology modeling and the best one was subjected to further refinement using the following procedures: (i) distance restraints were applied between the appropriate heavy and hydrogen atoms; (ii) minimization of the ligand position was performed with flexible side chain atoms of the interacting residues; (iii) minimization with flexible residues, which were closer than 4.5 Å from any ligand atom and restraints applied remained intact; and (v) restraints were removed and final minimization was performed. During the minimization steps, the MMFF94x force field was applied and the gradient was set to 0.05 kcal/mol Å2, and the protein was protonated at pH 7.4. Then, the molecular mechanics generalized Born interaction (MM/GBVI) energy between the modeled propionyl-CoA and the PccB complex was calculated, which comprises van der Waals, Coulomb, and generalized Born implicit solvent interaction energies [44]. For comparison, the complex between acetyl-CoA and PccB was also investigated. As the structure of acetyl-CoA closely resembles the propionyl-CoA, the binding of acetyl-CoA to PccB was modeled by reducing the propionyl moiety of the propionyl-CoA to an acetyl moiety, while keeping the remaining ligand atoms in place. After this minor structure perturbation, the above-mentioned four steps of refinement were followed. In the end, the MM/GBVI energy of the modeled acetyl-CoA and the PccB complex was reported.

Construction of PCC mutants
We constructed the site-directed mutations of carboxyltransferase (CT) PccC of PCC in L. interrogans according to the manufacturer’s instruction (Toyobo, Osaka, Japan). The primers were shown in Supplementary Table 4.

Results
Enzymatic activities of both ACC and PCC were detected in crude cell extracts of laboratory cultured L. interrogans strain 56601
Because genes encoding putative subunits of both ACC and PCC were annotated in the genome of L. interrogans serovar Lai [27], their activities against the substrate of acetyl-CoA or of propionyl-CoA were assayed in the crude cell extract of L. interrogans 56601 cultured. The specific activity of ACC detected was (2.00 ± 0.17) × 10⁻⁴ μmol/min mg protein, while that for PCC was (231.91 ± 10.06) × 10⁻⁴ μmol/min mg protein. This result indicated that the genome of L. interrogans 56601 indeed encoded acyl-CoA enzymes functional with ACC and PCC activities although more than 100-fold difference in their relative specific activities was unexpected.
Protein structural domain analysis and gene linkage analysis of the five putative acyl-CoA subunits revealed two different types of ACC/PCC

According to our recently updated annotation [45], two types of acyl-CoA carboxylases are likely encoded by the genome of Leptospira interrogans strain 56601 [Fig. 1(A)]. The LA_2432 may encode a protein composed of a biotin carboxylase (BC) domain fused with a biotin carboxyl carrier (BCCP) domain, and LA_2433 may encode a CT domain [Fig. 1(A)]. Because these two genes are closely linked and co-transcribed as a cistron as indicated by reverse transcription (RT)-PCR analysis [Fig. 1(B)], they are likely an acyl-CoA carboxylase holoenzyme composed of characteristic two subunits (BC/BCCP and CT, respectively) [46]. In contrast, LA_2736, LA_2735, and LA_3803 genes may encode a BC, a BCCP, and a CT, respectively [Fig. 1(A)]. It is also shown that LA_2736 and LA_2735 are closely linked and co-transcribed as a cistron [Fig. 1(B)]. Although LA_3803 gene is located on the genome far away from the LA_2736-LA_2735 cistron, and must be transcribed independently, these three genes are likely expressed at the same level (Table 1) if the expression data of whole genome transcriptome and proteome were considered in combination [21,45]. Therefore, we propose that these three genes may encode an acyl-CoA carboxylase holoenzyme composed of the three characteristic single domain subunits [46].

Heterologous co-expression of Leptospira interrogans acyl-CoA carboxylase subunit genes may reconstitute their ACC or PCC holoenzyme activities

Based on the above-mentioned analysis, the two predicted L. interrogans acyl-CoA carboxylase systems were reconstituted in E. coli via co-expression of the genes encoding the corresponding subunits. The LA_2432-LA_2433 cistron was cloned into the pRSFDuet-1 vector forming the expression plasmid pRDUBC, while the LA_3803 gene and the LA_2735-LA_2736 cistron were both cloned into the pRSFDuet-1 vector forming the expression plasmid pRDUPBC. These plasmids were transformed into E. coli BL21 (DE3) individually to express the recombinant proteins. Cell-free extract was prepared as describe above and the activities of both ACC and PCC were readily detected (Table 2). These results confirmed that the gene compositions for the two acyl-CoA carboxylases predicted by sequence analysis. In addition, because these two holoenzymes were significantly different in their relative catalytic activities against different substrates, acetyl-CoA versus propionyl-CoA, the LA_2432-LA_2433 encoded holoenzyme was tentatively designated ACC with subunits PccB (LA_2432) and PccC (LA_2433), while the LA_3803 and LA_2735-LA_2736-encoded holoenzyme was tentatively designated ACC with subunits AccP (LA_2735), AccB (LA_2736), and AccC (LA_3803). It should be noticed that there is no PCC in E. coli, while the activity of ACC is undetectable directly by carboxylation of acetyl-CoA in E. coli either [43,47–49]. Therefore, the enzyme activities detected should be attributed to the holoenzymes of ACC and PCC encoded by the corresponding L. interrogans genes.

Purification and kinetics analysis of Leptospira interrogans ACC and PCC revealed their different characteristics in substrate preferences

Owing to its extremely unstable property, we failed to purify the heterologously expressed L. interrogans ACC encoded by accPBC. However, its apparent kinetic parameters were determined as around 800 μM against acetyl-CoA and around 2200 μM against propionyl-CoA, using the crude cell extracts. Although the substrate preference of this holoenzyme is not significantly different between the two substrates, acetyl-CoA is apparently a more preferred one and the ACC designation was confirmed.

The heterologously expressed L. interrogans PCC encoded by pccBC was co-purified by Ni²⁺-NTA column (~81.55% purity by SDS-PAGE) [Fig. 2(A)]. The PccBC complex was identified by linear ion trap quadrupole (LTQ) [Fig. 2(C,D) and Supplementary Table 5] with a molecular weight slightly smaller than 669 kDa [Fig. 2(B)]. A quaternary structure of α₄β₄ dimers is suggested as the 1:1 stoichiometry for the subunits composition of ACC and PCC currently aware about [35,41,50,51].

The kinetic parameters of the partially purified PccBC complex were measured (Table 3). The apparent Km value against propionyl-CoA is 110 ± 13 μM, with a kcat of 2.86 ± 0.05 S⁻¹. The apparent Km value against acetyl-CoA
is 4.6 ± 0.5 mM, with a $k_{\text{cat}}$ of 0.66 ± 0.03 S$^{-1}$. This substrate preference confirmed that the holoenzyme is a PCC.

Table 1 Microarray and mass spectrometry data for genes involved in partial 3-hydroxypropionate pathway from acetyl-CoA to succinyl-CoA in *Leptospira interrogans* strain 56601

| Gene ID | Annotation | EC number | COGs          | Microarray data | MS data |
|---------|------------|-----------|---------------|-----------------|--------|
| LA2432  | Propionyl-CoA carboxylase | EC 6.4.1.2 | COG4770I      | 2279.00         | 1.7604 |
| LA2433  | Acetyl-CoA carboxylase     | EC 6.4.1.3 | COG4770I      | 670.83          | 0.2328 |
| LA2735  | Malonate-semialdehyde dehydrogenase | EC 1.2.1.18 | COG0136E    | 1906.00         | 1.3190 |
| LA4138  | 3-hydroxypropionate dehydrogenase (NADP$^+$) | EC 1.1.1.298 | COG1250I    | 4054.00         | 5.7626 |
| LA4254  | 3-hydroxypropionate-CoA ligase | EC 6.2.1.-    | COG0365I     | 1104.20         | 0.4946 |
| LA1198  | 3-hydroxypropionyl-CoA dehydratase | EC 4.2.1.-    | COG1024I    | 1932.00         | 1.3279 |
| LA0073  | Acryl-CoA reductase         | EC 1.3.1.84 | COG0604CR    | 1134.00         | 0.6807 |
| LA1417  | Methylmalonyl-CoA epimerase | EC 5.1.99.1 | –            | 1309.00         | 1.6124 |
| LA2956  | Methylmalonyl-CoA mutase    | EC 5.4.99.2 | COG1884I     | 440.03          | 0.2829 |
| LB074   | Malonate-semialdehyde dehydrogenase | EC 1.2.1.18 | COG1064R    | 759.20          | 0.1196 |
| LB273   | Methylmalonyl-CoA mutase    | EC 5.4.99.2 | COG1884I     | 2627.00         | 1.5955 |
| LB274   | Methylmalonyl-CoA mutase    | EC 5.4.99.2 | COG1884I     | 951.43          | 0.7290 |
| LB274   | Methylmalonyl-CoA mutase    | EC 5.4.99.2 | COG1884I     | 1333.00         | 0.6368 |

The numbers in ‘microarray data’ and ‘MS data’ column are the transcriptional signals and the calculated protein abundance, respectively. The average expression signal of all the genes on the microarray was 1038.74, and the average protein abundance of whole cell extracts was 1.0877.

Table 2 Heterologous expression of *Leptospira interrogans* acyl-CoA carboxylases in *Escherichia coli* and reconstitution of enzyme activity in cell-free extract

| Protein | Plasmids | ACCase (mU/mg protein)$^a$ | PCCase (mU/mg protein) |
|---------|----------|---------------------------|------------------------|
| PccB, PccC | pRDUBC  | 57.33 ± 1.58              | 2641.07 ± 307.36         |
| AccP, AccB, | pRDUPBC | 169.76 ± 15.37            | 149.61 ± 21.99          |

$^a$10–30 cpm equivalent to 0.02–0.06 mU.

is 4.6 ± 0.5 mM, with a $k_{\text{cat}}$ of 0.66 ± 0.03 S$^{-1}$. This substrate preference confirmed that the holoenzyme is a PCC.

Structural simulation and identification of Y430 and A431 as the potentially critical amino acid residues located at the bottom of the substrate binding pocket in PCC

CT Pcc is the catalytic subunit of PCC. The crystal structure of CT PccB of the *S. coelicolor* PCC with substrate propionyl-CoA bound (PDB ID: 1XNY) [52] provided a structural scenario to explain the substrate specificity of the enzyme. Because the amino acid sequences of the PccB from *S. coelicolor* and the PccC from *L. interrogans* shared 34% identity, the 3D structural model of PccC was simulated using the MOE software based on the *S. coelicolor* PccB template. The substrate-binding pocket of PccC was noticed in the simulated structural model and sequence comparison indicated that most of the amino acid residues surrounding the pocket were highly conserved, except for Y430 and A431 located at the bottom of the acyl-CoA-binding pocket (Fig. 3).

The *S. coelicolor* PCC has $K_m$ of 76.5 $\mu$M against propionyl-CoA but no activity towards acetyl-CoA. This extraordinary stringent substrate specificity was accounted by the side-chain size of the amino acid residue Asp located at the bottom of the substrate binding pocket [39]. To understand the molecular basis of substrate specificity of the *L. interrogans* PCC, we performed a site-directed mutagenesis experiment. Single-point mutation of A431I, A431C, A431D, A431L and a double mutation of Y430H-A431I were introduced into the *L. interrogans* PCC. All five purified mutated PCCs were soluble, and subjected for further investigation. The kinetic parameters of the mutants were compared with that of the wild type, as shown in Table 3. A double mutation of Y430H-A431I and two single mutations of A431D and A431L showed complete loss of the activity. Two mutants of A431C and A431I resulted in decrease of affinity for PCC towards to propionyl-CoA, but only minor variations toward acetyl-CoA.
Figure 2 Co-purification of expressed PccBC operon from a Ni²⁺ affinity column and the determination of its native molecular weight

(A) Eluted with buffer A containing 250 (1, 2, 3, 4, and 5) mM imidazole. SM, standard molecular mass markers. Aliquots of the fractions were subjected to 12% SDS-PAGE and Coomassie blue stained. (B) After dialysis and concentration, the elution was subjected to 6% native-PAGE. SM, standard molecular mass markers. (C) Characteristic peptide (ALSFYQELAK) MS–MS schematic diagram of PccB by LTQ. (D) Characteristic peptide (ELLNKPLLGGGVDR) MS–MS schematic diagram of PccC by LTQ.
Discussion

Experimental characterization of ACC and PCC from L. interrogans strain 56601 facilitates more precise annotation of their homologous genes in other Leptospira species

In this paper, two key enzymes potentially involved in an annotated partial 3-hydroxypropionate pathway for anaplerotic CO₂ assimilation in L. interrogans [27,45], the tri-subunit ACC composed of AccP (LA_2735), AccB (LA_2736), and AccC (LA_3803), and the bi-subunit PCC composed of PccB (LA_2432) and PccC (LA_2433) were experimentally characterized. Although we failed to purify the ACC to homogeneity, its substrate preference towards acetyl-CoA over propionyl-CoA was experimentally determined by measuring the apparent \( K_m \) values of corresponding substrates. In contrast, the PCC was purified and thoroughly characterized not only at the enzyme kinetics level but also at the structure–function mechanistic level, which probably accounts for its substrate preference as that will be discussed later.

Besides the large, multi-domain enzyme found in the endoplasmic reticulum of most eukaryotes [53–55], ACC is an oligomeric enzyme found in most prokaryotes and in the chloroplasts of most plants [56–60], and they could be classified based on their oligomeric special structures [46]. The bacterial type is composed of four subunits of BC, BCCP, CTₐ, and CTₐ [56]; the actinomycetic type is composed of α and β subunits, each containing the BC and BCCP domains and the CT domain, respectively [39,61]; and the archaea type is composed of BC, BCCP, and CT subunits [35]. Therefore, based on their subunit compositions, the ACC and PCC in L. interrogans strain 56601 can be designated archaea and actinomycetic types, respectively. In addition, when we search into the genomes of other leptospiral species, we did identify homologous genes encoding enzymes of both types in all of these species although in L. borgpetersenii serovar Hardjo bovis, the accC genes of the two sequenced strains are both frameshift mutants (pseudogenes) caused by different kinds of small indels (Supplementary Fig. 1 and Table 4). This more precise annotation for genes encoding ACC and PCC inferred that the previously proposed partial 3-hydroxypropionate pathway for anaplerotic CO₂ assimilation might be universal in leptospires in general. On the other hand, the identification of accC pseudogenes in L. borgpetersenii may recall the very low activity of L. interrogans ACC detected in the crude extract of the

| Enzymes | Acetyl-CoA | | Propionyl-CoA | |
|---------|------------|-----------------|-----------------|
|         | \( K_m (\mu M) \) | \( k_{cat} (S^{-1}) \) | \( k_{cat}/K_m (M^{-1} S^{-1}) \) | \( K_m (\mu M) \) | \( k_{cat}(S^{-1}) \) | \( k_{cat}/K_m (M^{-1} S^{-1}) \) |
| Wild-type | (4.6 ± 0.5) × 10³ | 0.66 ± 0.03 | (1.44 ± 0.17) × 10² | (1.1 ± 0.1) × 10² | 2.86 ± 0.05 | (2.60 ± 0.32) × 10⁴ |
| A431I   | (5.0 ± 0.9) × 10³ | 1.4 ± 0.2    | (2.74 ± 0.57) × 10² | (1.3 ± 0.2) × 10³ | 0.47 ± 0.04 | (3.67 ± 0.75) × 10² |
| Y430H, A431I | ND   |              |                 | ND               |              |                     |
| A431C   | (4.8 ± 0.5) × 10³ | 4.00 ± 0.29  | (8.26 ± 1.10) × 10² | 240 ± 31        | 1.46 ± 0.10  | (6.09 ± 0.89) × 10³ |
| A431D   | ND       |              |                 | ND               |              |                     |
| A431L   | ND       |              |                 | ND               |              |                     |

ND, not detectable.

Figure 3 Sequence alignment of CTs subunits from Leptospira interrogans and other bacteria, against that from human The abbreviations used for different bacteria species are: sco, Streptomyces coelicolor (the 3D structure has been determined, PDB ID: 1XNY); mxa, Myxococcus xanthus; mse, Metallosphaera sedula; abr, Acidaminobacter brierleyi; mrv, Mycobacterium tuberculosis; hsa, Homo sapiens (human); ser, Streptomyces erythreus; lil, Leptospira interrogans; sve, Streptomyces venezuelae; tte, Thermoanaerobacter tengcongensis. Amino acid residues marked with red underlines represented the conserved binding sites and catalytic sites.
bacterium. Therefore, later in this section, we will further discuss issues of whether gluconeogenesis via anaplerotic CO₂ assimilation is essential for the growth of leptospires; whether the proposed partial 3-hydroxypropionate pathway does exist and whether it is the major means of gluconeogenesis in the bacterium.

The substrate preference properties of \textit{L. interrogans} ACC and PCC are apparently different from their counterparts in other prokaryotes

Based on the tri-subunit composition, the ACC of \textit{L. interrogans} can be categorized as the archaea type. Its substrate affinity indicated by its apparent \(K_m\) values of \(\sim 800 \mu M\) toward acetyl-CoA and \(\sim 2.2 \mu M\) toward propionyl-CoA, is at least one order higher than that of the ACC enzymes found in other prokaryotes [35,39,62]. For instance, in \textit{Archaea}, the ACC of \textit{Metallosphaera sedula} has its \(K_m\) of 60 \(\mu M\) and 70 \(\mu M\) toward acetyl-CoA and propionyl-CoA, respectively, while the ACC of \textit{Acidianus brierleyi} has its \(K_m\) of 170 and 100 \(\mu M\) toward acetyl-CoA and propionyl-CoA, respectively. Even the actinomycetic type ACC of \textit{S. coelicolor} A3(2) has its \(K_m\) of 97.9 \(\mu M\) and 89.4 \(\mu M\) toward acetyl-CoA and propionyl-CoA, respectively. Although the \textit{L. interrogans} PCC, as a bi-subunit actinomycetic-type enzyme, with its \(K_m\) value of 110 ± 13 \(\mu M\) toward propionyl-CoA, is comparable to that of \textit{S. coelicolor} with its \(K_m\) of 76.5 \(\mu M\), the latter has no detectable ACC activity [39] but the \textit{L. interrogans} PCC does have its \(K_m\) value of 4.6 ± 0.5 \(\mu M\) toward acetyl-CoA. Although this low but detectable ACC activity might complement for the pseudogene defect of \textit{accC} in \textit{L. borgpetersenii}, its physiological significance is yet to be tested.

In order to understand the structural basis for substrate preference of \textit{L. interrogans} PccC, we used the \textit{S. coelicolor} PccB as a template to simulate the 3D structure model of PccC because both of them belong to the same PCC category and with the same CT activity. Most of the amino acid residues surrounding the substrate-binding pocket of PccC seemed to be conserved except for A431. We thus assumed that as PccB, the A431 of PccC may affect its substrate preference. We constructed four single-point mutants of A431I, A431C, A431D, A431L, and a double mutation of Y430H-A431I in PccC. These mutated enzymes are all soluble and stable so that their global conformations seem to be well maintained. The individual A431I and A431C substitutions in PCC resulted in decreased affinity toward propionyl-CoA but almost no effect on acetyl-CoA, whereas the other two mutants of A431D and A431L led to the complete loss of activity (Table 3). Therefore, the amino acid properties of A431 may help to define the shape of the acyl-CoA-binding pocket. In addition, comparing the kinetic parameters of these mutants with that of the wild-type enzymes (Table 3), the complete loss of activity by the Y430H-A431I double mutant may further indicate that Y430 is essential in maintaining the enzyme activity although other residues surrounding the active center may also be important for determining the substrate selectivity.

Two important enzymes, ACC and PCC, involved in the proposed partial 3-hydroxypropionate pathway for net gluconeogenesis from acetyl-CoA via anaplerotic assimilation of CO₂ are proved to be active in \textit{L. interrogans}

Previous studies indicated that neither pathogenic nor saprophytic leptospires could use glucose or any other kinds of sugars [27,63]. When cultured in vitro, they can grow in minimal medium EMJH, in which, although four potential carbon/energy sources were supplied, only the long-chain fatty acid, Tween 80 rather than glycerol, sodium pyruvate and sodium acetate, is essential [30, data

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Table 4 Re-annotated genes encoding PCC and ACC in five \textit{Leptospira} strains with complete genome sequences

| Gene Product name | PCC:BC and BCCP subunit | PCC:CT subunit | ACC:BCCP subunit | ACC:BC subunit | ACC:CT subunit |
|-------------------|--------------------------|----------------|-----------------|----------------|----------------|
| \textit{Leptospira interrogans} serovar Lai 56601 | LA_2432 | LA_2433 | LA_2735 | LA_2736 | LA_3803 |
| \textit{L. interrogans} serovar Copenhageni Fiocruz L1 130 | LIC11518 | LIC11517 | LIC11278 | LIC11277 | LIC10443 |
| \textit{Leptospira biflexa} serovar Patoc Patoc 1 Ames | LBF_1527 | LBF_1526 | LBF_0539 | LBF_0538 | LBF_0501 |
| \textit{L. biflexa} serovar Patoc Patoc 1 Paris | LEPBI_11578 | LEPBI_11577 | LEPBI_10558 | LEPBI_10557 | LEPBI_1052 |
| \textit{Leptospira borgpetersenii} serovar Hardjo bovis JB197 | LBJ_1817 | LBJ_1818 | LBJ_1712 | LBJ_1713 | LBJ_0370 |
| \textit{L. borgpetersenii} serovar Hardjo bovis L550 | LBL_1466 | LBL_1465 | LBL_1931 | LBL_1932 | LBL_2707c |

*aGenes functioning in step VII.

*bGenes functioning in step I.

*cPseudo gene.
not shown]. It was also reported that, within the family of Spirochaetales, unlike the Spirochaeta, most species of Leptospira were unable to synthesize long-chain fatty acids de novo or to elongate the organic acids provided [64]. Therefore, it is reasonable to hypothesize that acetyl-CoA, generated from catabolic utilization of long-chain fatty acids, besides generating energy via oxidative phosphorylation, must be the major precursor for carbon skeleton supply in anabolism of leptospires via gluconeogenesis, which is essential for the growth.

However, when the genome of L. interrogans serovar Lai was firstly annotated, neither of the two key enzymes of the glyoxylate pathway, isocitrate lyase and malate synthase, were identified and we failed to detect their activities in the cell crude extract either [27]. Further genomic analysis of the saprophytic L. biflexa [28] failed to identify genes encoding these two key enzymes despite the earlier report about the detection of their biochemical activities [26]. Therefore, alternative pathways for gluconeogenesis ought to be identified to account for the growth of leptospires on minimal EMJH medium.

Gene-encoding enzymes likely to be involved in the carboxylation of acetyl-CoA to succinyl-CoA via a 3-hydroxypropionate pathway proposed in some autotrophic bacteria [31] were recognized in the genome of L. interrogans and a partial 3-hydroxypropionate pathway for net gluconeogenesis metabolism by anaplerotic CO₂ assimilation with net conversion of acetyl-CoA to glucose and other saccharides was hypothesized [27]. As shown in this report, gene expression profiling and quantitative proteomic data mining provided convincing evidence that all of these genes did expressed in L. interrogans during in vitro culturing (Table 1). In this study, as an initial biochemical analysis effort, we demonstrated that both ACC and PCC were active in L. interrogans and the enzymatic property of the bi-subunit PCC was characterized. On the other hand, we noticed that the total activity of ACC in L. interrogans was not only significantly lower than that of PCC, but also too low (0.2 nmol/min mg protein) to meet the physiological demand for carbon skeleton matching with the growth rate (12-h doubling time [65]) of L. interrogans in EMJH minimal medium albeit its in vivo activity might be higher than it has been detected in vitro. Therefore, final proof of this hypothetical partial 3-hydroxypropionate pathway requires not only fully experimental characterization of the L. interrogans ACC, both in vitro and in vivo, as well as identification of other related enzymes such as malonate-semialdehyde dehydrogenase and acrylyl-CoA reductase (Table 1), but also the measurement of dynamics for the flux of quantitative incorporation of CO₂ into cellular metabolites in vivo. In that direction, the previous observation about CO₂ as an essential element for in vitro growth of L. interrogans [29,30] should be noticed as the initial physiological analysis effort towards elucidating the mechanism of leptospiral gluconeogenesis via anaplerotic CO₂ assimilation.

**Supplementary Data**

Supplementary data are available at ABBS online.

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