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Host-Microbial Interactions | Full-Length Text

The mitochondrial carboxylase PCCA interacts with *Listeria monocytogenes* phospholipase PlcB to modulate bacterial survival

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ABSTRACT *Listeria monocytogenes*, a prominent foodborne pathogen responsible for zoonotic infections, owes a significant portion of its virulence to the presence of the phospholipase PlcB. In this study, we performed an in-depth examination of the intricate relationship between *L. monocytogenes* PlcB and host cell mitochondria, unveiling a novel participant in bacterial survival: the mitochondrial carboxylase propionyl-coenzyme A carboxylase (PCCA). Our investigation uncovered previously unexplored levels of interaction and colocalization between PCCA and PlcB within host cells, with particular emphasis on the amino acids 504–508 of PCCA, which play a pivotal role in this partnership. To assess the effect of PCCA expression on *L. monocytogenes* proliferation, PCCA expression levels were manipulated by siRNA-si-PCCA or pCMV-N-HA-PCCA plasmid transfection. Our findings demonstrated a clear inverse correlation between PCCA expression levels and the proliferation of *L. monocytogenes*. Furthermore, the effect of *L. monocytogenes* infection on PCCA expression was investigated by assessing PCCA mRNA and protein expression in HeLa cells infected with *L. monocytogenes*. These results indicate that *L. monocytogenes* infection did not significantly alter PCCA expression. These findings led us to propose that PCCA represents a novel participant in *L. monocytogenes* survival, and its abundance has a detrimental impact on bacterial proliferation. This suggests that *L. monocytogenes* may employ PlcB-PCCA interactions to maintain stable PCCA expression, representing a unique pro-survival strategy distinct from that of other intracellular bacterial pathogens.

IMPORTANCE Mitochondria represent attractive targets for pathogenic bacteria seeking to modulate host cellular processes to promote their survival and replication. Our current study has uncovered mitochondrial carboxylase propionyl-coenzyme A carboxylase (PCCA) as a novel host cell protein that interacts with *L. monocytogenes* PlcB. The results demonstrate that PCCA plays a negative regulatory role in *L. monocytogenes* infection, as heightened PCCA levels are associated with reduced bacterial survival and persistence. However, *L. monocytogenes* may exploit the PlcB-PCCA interaction to maintain stable PCCA expression and establish a favorable intracellular milieu for bacterial infection. Our findings shed new light on the intricate interplay between bacterial pathogens and host cell mitochondria, while also highlighting the potential of mitochondrial metabolic enzymes as antimicrobial agents.

KEYWORDS *Listeria monocytogenes*, phospholipase PlcB, mitochondria propionyl-CoA carboxylase (PCCA), bacterial proliferation, host-pathogen interaction

Listeria monocytogenes is an opportunistic intracellular zoonotic bacterium that poses a significant threat to human health. This bacterial pathogen is able to cross

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the intestinal barrier, the blood-brain barrier, and the fetoplacental barrier and cause gastroenteritis in healthy individuals, meningitis and meningoencephalitis in immunocompromised individuals, as well as abortions in pregnant women (1–3). *L. monocytogenes* is widespread in nature and can grow at low and high temperatures, dryness, extreme pH, and high salinity environment, which allow it to persist in food-manufacturing sites for several years (4, 5). After ingestion of highly contaminated food, *L. monocytogenes* is able to cross the intestine invading phagocytic and non-phagocytic cells (6). After internalization, the secreted pore-forming toxin listeriolysin O (LLO) and two phospholipases (PlcA and PlcB) rapidly mediate *L. monocytogenes* to escape from the phagosome into the cytosol, which facilitates the bacteria survival, intracellular replication, and eventual spread from primarily infected cells to neighboring cells of host (7, 8). In the recipient adjacent cells, *L. monocytogenes* is entrapped in a double-membrane vacuole where PlcB causes the dissolution of the inner membrane and plays a key role in vacuolar escape and cell-to-cell spread. Phospholipases play an important role in the absence of LLO. In LLO-deficient strains of *L. monocytogenes*, PlcB is required for rupture of primary vacuoles in human epithelial cell lines (9, 10).

Intracellular bacterial pathogens have evolved multiple strategies to manipulate host cellular processes and subvert the immune response. One such strategy is to target host cell mitochondria, which are involved in critical cellular functions such as energy production, apoptosis, calcium signaling, and innate immunity (11). Pathogenic bacteria, like *L. monocytogenes*, *Legionella pneumophila*, *Shigella flexneri*, and *Mycobacterium tuberculosis*, can manipulate mitochondrial dynamics to promote intracellular replication, evade host immune responses, and establish chronic infections. For instance, *L. monocytogenes* secretes listeriolysin O, a pore-forming toxin that disrupts mitochondrial membrane potential and promotes bacterial survival by inhibiting host cell apoptosis (12, 13). Similarly, *L. pneumophila* secretes effector proteins that target mitochondrial fission and fusion machinery, resulting in fragmentation of the mitochondrial network and inhibition of autophagy (14). *S. flexneri* and *M. tuberculosis* also target mitochondrial function and dynamics to promote their survival and replication within host cells (15, 16). These findings highlight the importance of mitochondria in host-pathogen interactions and the potential to target mitochondrial pathways as a novel therapeutic approach for intracellular bacterial infections.

Propionyl-coenzyme A carboxylase (PCCA) is an enzyme that catalyzes the carboxylation of propionyl-coenzyme A (CoA) to methylmalonyl-CoA, which contributes to the replenishment of tricarboxylic acid (TCA) cycle intermediates. PCCA is encoded by *pcca* and *pccb*. The Both1 N and C terminals of PCCA are necessary for holocarboxylase synthase interaction, and variants in these domains can perturb this interaction and cause disease (17). Of note, deficiency of *pcca* can significantly reduce mitochondrial content and mitochondrial membrane potential and significantly increase mitochondrial matrix superoxide burden, culminating in reduced animal lifespan (18). Recent studies have indicated the existence of active interactions between pathogen infection and host mitochondria. For instance, later in Enteropathogenic *Escherichia coli* infection, secreted protein EspH-dependent increase in FIS1 can result in significant mitochondrial fragmentation and host cell death, facilitating pathogen dispersal (19). The mitochondrial motrix protein ERAL1 can be released to the cytosol to facilitate antiviral immunity during RNA virus infection (20). PCCA is a mitochondrial carboxylase and loosely bound to the mitochondrial inner membrane-matrix subcellular fraction (21). Despite PCCA involvement of PCCA in energy metabolism, whether PCCA is a target protein involved in pathogen infection is not well understood.

In summary, our study investigated the impact of *L. monocytogenes* infection on host cell mitochondria and identified a novel factor, PCCA, which is involved in bacterial survival. We found that upregulation of PCCA levels significantly inhibited *L. monocytogenes* survival, indicating a potential new target for controlling *Listeria* infections. However, our results also suggest that *L. monocytogenes* may exploit the interaction between PlcB and PCCA to maintain stable PCCA expression and create a favorable

intracellular environment for bacterial infection. These findings deepen our understanding of the intricate interplay between host cells and bacterial pathogens and provide new insights into the mechanisms underlying *Listeria pathogenesis*. Future studies should explore the potential of targeting PCCA as a therapeutic strategy for controlling *Listeria* infections.

RESULTS

L. monocytogenes PlcB interacts with the host PCCA

In the yeast two-hybrid assay, the pGADT7-PCCA plasmid was used to verify the interaction between PlcB and PCCA. The results showed that only the colonies carrying the pGADT7-PCCA and pGBKT7-BD-PlcB plasmids, as well as the positive control group, were able to grow normally in the quadruple dropout medium (QDO) plates and blue in the QDO/X/A plates, while all the plasmids were able to grow normally in the double dropout medium (DDO) plates (Fig. 1A). In addition, the results of the co-immunoprecipitation (Co-IP) assay showed that the PCCA protein carrying the HA tag could be successfully detected using Myc-Tag (Fig. 1B). Immunofluorescence analysis also indicated that the PCCA protein labeled with green fluorescence colocalized with the PlcB protein labeled with red fluorescence, as the green fluorescence completely overlapped with the red fluorescence (Fig. 1C). These findings strongly suggest that PCCA and PlcB interact and colocalize within cells.

Identification of key interaction region between PCCA and PlcB proteins

To identify the key structural domains in which PlcB specifically binds to PCCA, eukaryotic expression plasmids of truncated PCCA proteins were constructed. Serial numbers of the PCCA eukaryotic expression plasmids are provided in Table S1. The truncation strategies for PCCA eukaryotic expression plasmids are shown in Fig. 2A. The results of the Co-IP assay demonstrated that the 389–508 amino acids of PCCA contained a key interaction region with the PlcB protein (Fig. 2B). To further narrow down the specific amino acid residues involved in this interaction, truncated plasmids were constructed using an amino acid truncation strategy. The results revealed that amino acids 504–508 of PCCA were responsible for interacting with the PlcB protein (Fig. 2C and D).

Overexpression of PCCA inhibits bacterial intracellular proliferation

To evaluate the effect of PCCA expression on the proliferation of *L. monocytogenes*, HeLa cells were transfected with the HA-PCCA plasmid, and mRNA transcription and protein expression levels were analyzed. Total RNA and secretory proteins were collected from HeLa cells transfected with the HA-PCCA plasmid and subsequently used for mRNA transcription and protein expression analysis. The results showed that the mRNA transcription and protein expression levels of PCCA were significantly upregulated in cells transfected with the HA-PCCA plasmid compared with cells transfected with the empty parental plasmid ($P < 0.05$; Fig. 3A through C). Infection with the EGD-e strain showed that *L. monocytogenes* proliferation in PCCA overexpressed cells decreased significantly 6 h post infection ($P < 0.05$; Fig. 3D). These findings suggest that PCCA overexpression effectively inhibits *L. monocytogenes* proliferation.

Knockdown of PCCA promotes bacterial intracellular proliferation

The knockdown efficiency of siRNA-si-PCCA was assessed by measuring the mRNA transcription and protein expression levels of PCCA in the transfected cells using real-time quantitative PCR (RT-qPCR) and western blotting. The results showed that the mRNA transcription and protein expression levels of PCCA were significantly downregulated in cells transfected with siRNA-si-PCCA compared to those transfected with siCtrl ($P < 0.05$; Fig. 4A through C). Additionally, the proliferative ability of *L. monocytogenes* increased significantly in cells with inhibited PCCA at 6 h post infection ($P < 0.05$; Fig. 4D).

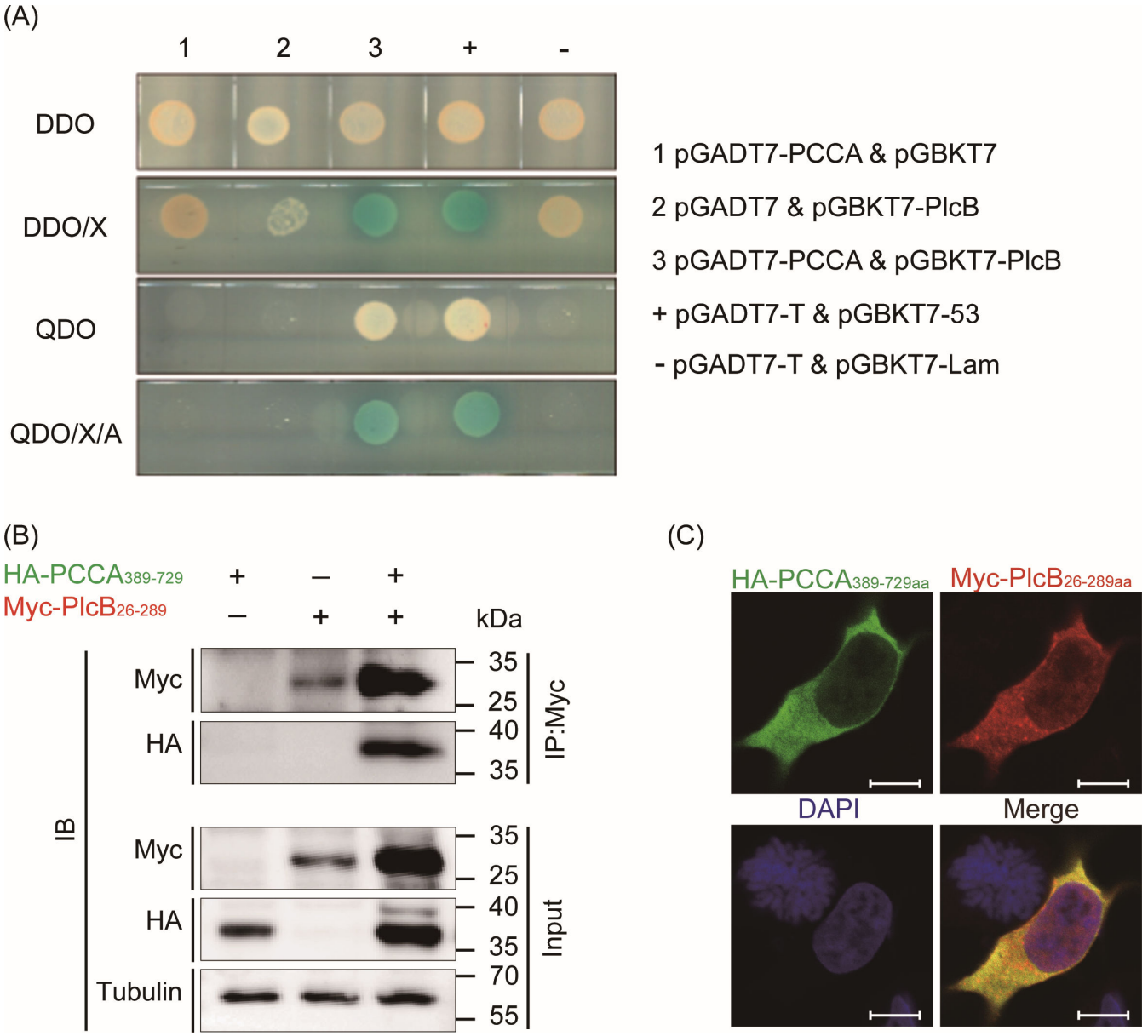

FIG 1 Interaction between *L. monocytogenes* PlcB and host PCCA. (A) Yeast two-hybrid revealed PCCA interaction with PlcB; DDO = double dropout medium; DDO/X = DDO with additional addition X-α-Gal (1:500); QDO = quadruple dropout medium; QDO/X = QDO with additional addition X-α-Gal (1:500) and Aureobasidin A (AbA; 1:2,500). (B) Co-IP assay identified the interaction between PCCA and PlcB. The abbreviations are IB for immunoblotted and IP for immunoprecipitated. (C) Immunofluorescence analysis showed colocalization of PCCA protein labeled with green fluorescence and PlcB protein labeled with red fluorescence. The scale bars mean 10 µm.

These results suggested that PCCA may play a negative regulatory role in the proliferation of *L. monocytogenes* in host cells.

L. monocytogenes infection did not affect PCCA expression

To further investigate the effect of *L. monocytogenes* infection on PCCA expression levels, the mRNA transcription and protein expression levels of PCCA in HeLa cells infected with either EGD-e or Δ*plcB* strains were measured. RT-qPCR and western blotting results showed no significant differences in the mRNA and protein expression levels of PCCA between cells treated with phosphate buffered saline (PBS) and cells infected with either *L. monocytogenes* strain ($P > 0.05$; Fig. 5A through C). Given that PCCA is mainly located in

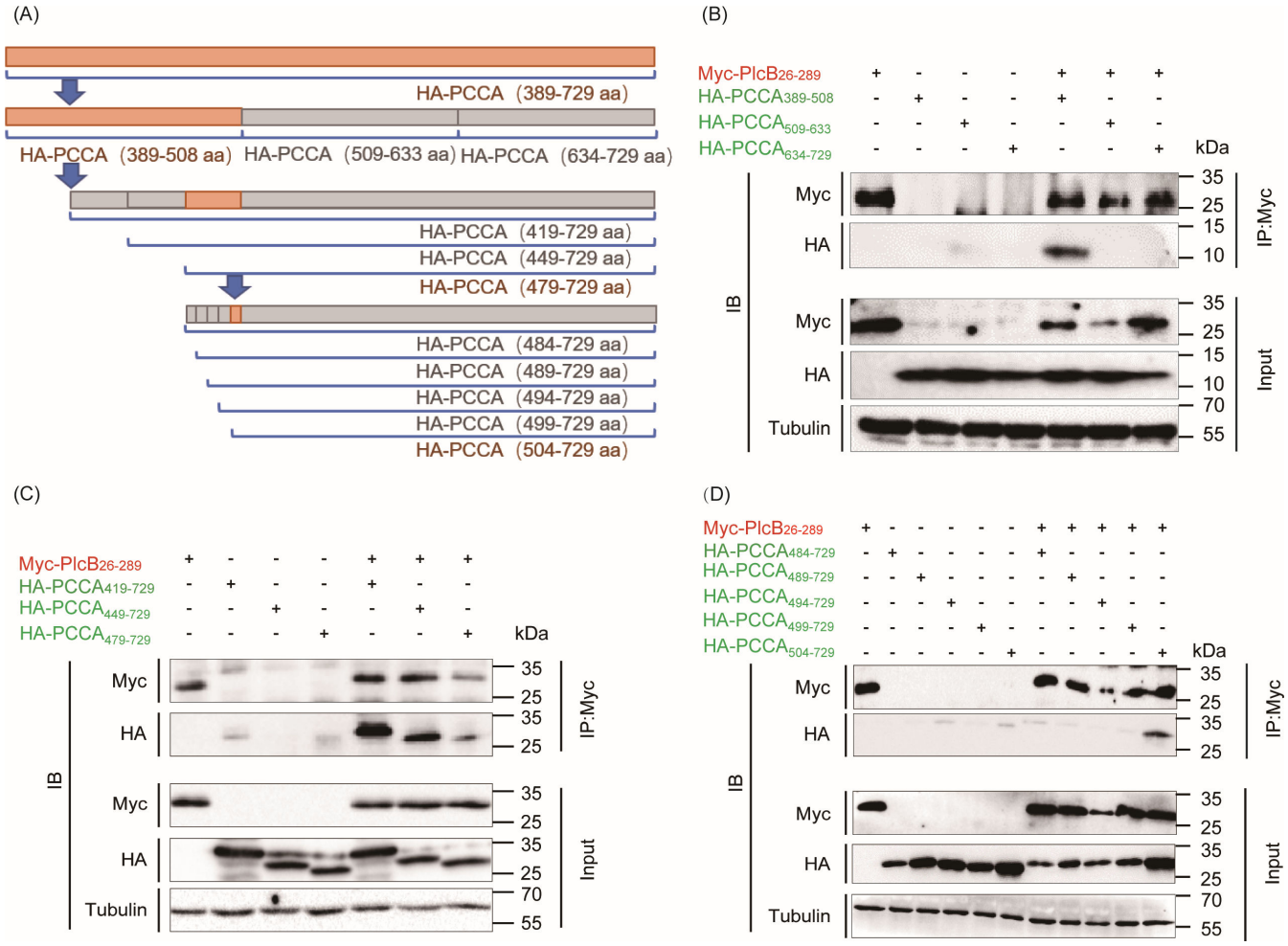

FIG 2 Identification of key interaction region between PCCA and PlcB proteins. (A) Illustration of the truncation strategies used for the eukaryotic expression plasmids of PCCA. (B) The segment comprising amino acids 389–508 of PCCA is identified as a crucial interaction domain with the PlcB protein. (C) A distinct key interaction region with the PlcB protein is located within amino acids 479–508 of PCCA. (D) The amino acids 504–508 of PCCA are specifically responsible for interaction with the PlcB protein.

the mitochondrial matrix, mitochondrial proteins from cells were collected to compare the changes in PCCA levels in mitochondria after infection with different strains. Similarly, the results showed that PCCA expression in mitochondria was not significantly affected by *L. monocytogenes* infection ($P > 0.05$; Fig. 5D and E). These findings suggest that the interaction between PlcB and PCCA may be skillfully exploited by *L. monocytogenes* during infection to maintain stable PCCA expression in the host. This, in turn, creates a favorable intracellular environment for *L. monocytogenes* infection.

DISCUSSION

Intracellular bacterial survival and multiplication depend on their ability to escape from the phagosome into the cytosol, which is a critical step in the complex and coordinated intracellular life cycle of *L. monocytogenes*. The escape of *L. monocytogenes* from double-membrane secondary vacuoles depends on the synergistic action of LLO, PlcA, and PlcB (22, 23). Previous studies have demonstrated that the phospholipases PlcA and PlcB play critical roles in the intracellular survival and replication of *L. monocytogenes*. Bacterial mutants lacking both phospholipases were mostly unable to dissolve the inner membrane of the secondary vacuole despite the presence of LLO (9). PlcB, in particular, was found to be essential for disintegrating the lipid membrane of the vacuole and the inner

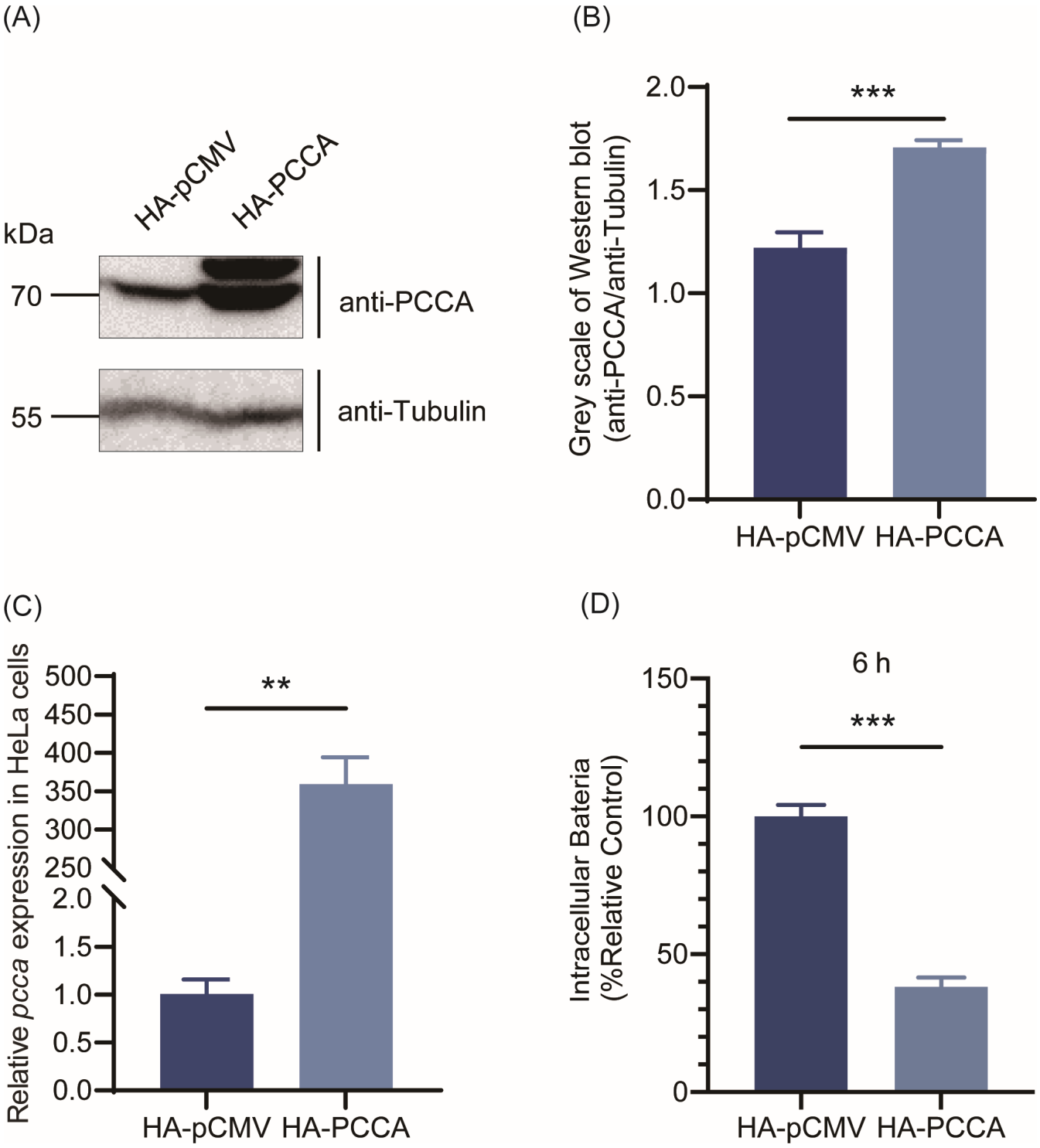

FIG 3 Overexpression of PCCA inhibits bacterial intracellular proliferation. (A) Western blot analysis of PCCA expression levels in HeLa cells transfected with HA-PCCA or HA-pCMV plasmids. (B) Gray value analysis of PCCA expression levels. (C) Real-time quantitative PCR (RT-qPCR) analysis of *pcca* transcript levels. (D) Percentage of intracellular bacteria at 6 h post-EGD-e infection. Data are expressed as the mean ± SD ($n = 3$). *$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

membrane of secondary vacuoles with a double membrane (9, 22). In this study, we used a Co-IP assay to identify host cell proteins that interact with PlcB and to investigate their potential role in *L. monocytogenes* survival and persistence. Our results identified mitochondrial carboxylase PCCA as a novel host cell protein that interacts with PlcB.

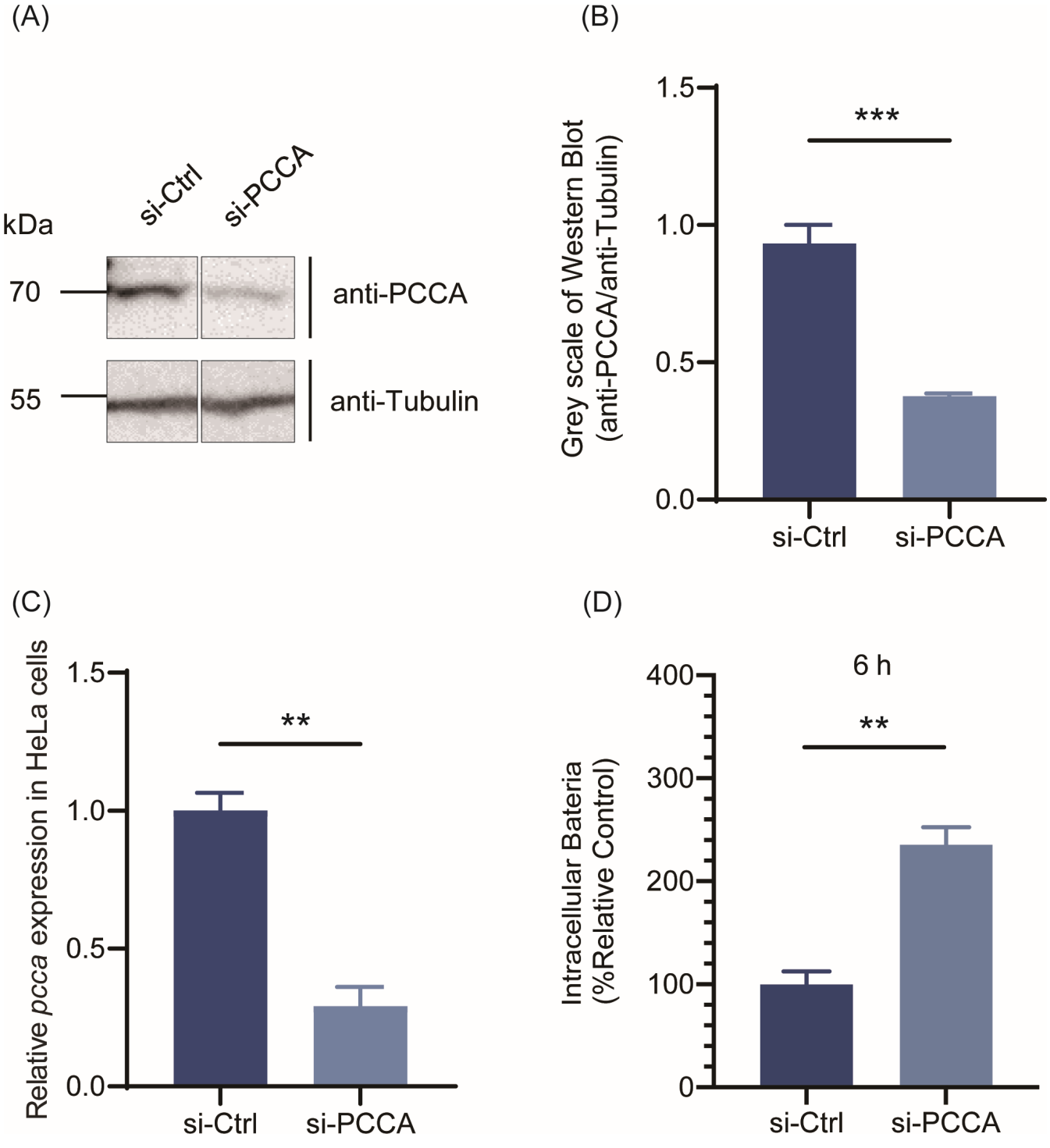

FIG 4 PCCA knockdown promotes bacterial intracellular proliferation. (A) Western blotting analysis of PCCA expression levels in HeLa cells transfected with si-PCCA or si-Ctrl. (B) Gray value analysis of PCCA expression levels. (C) RT-qPCR analysis of *pcca* transcript levels. (D) Percentage of intracellular bacteria at 6 h post-EGD-e infection. Data are expressed as the mean ± SD ($n = 3$). *$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$.

Furthermore, our findings suggest that PCCA negatively regulates *L. monocytogenes* infection, as an increased abundance of PCCA was found to be associated with reduced bacterial survival and persistence. This study sheds new light on the complex interactions between *L. monocytogenes* and its host cells, highlighting the importance of phospholipases and the novel role of PCCA in *L. monocytogenes* infections.

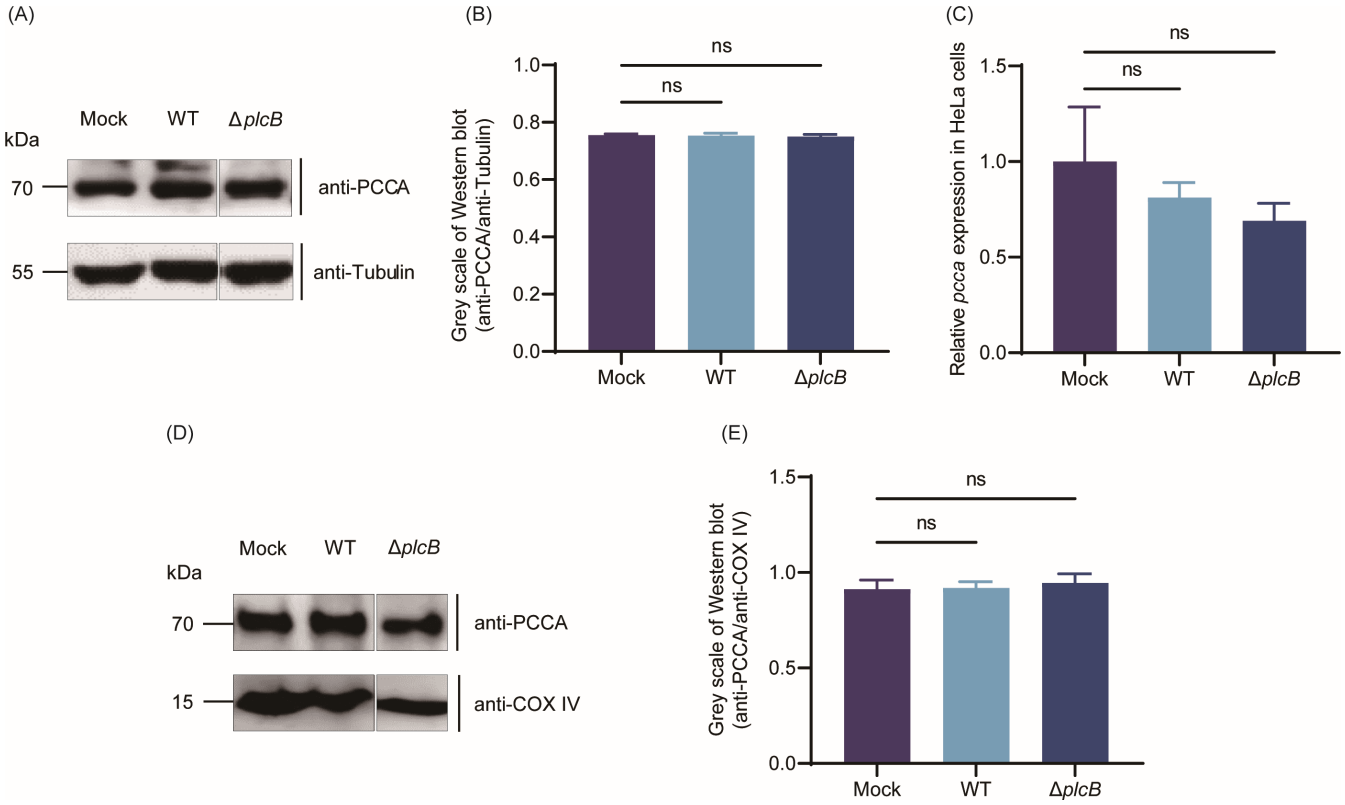

FIG 5 *L. monocytogenes* infection does not affect PCCA expression. (A) Western blotting analysis of PCCA expression in HeLa cells infected with EGD-e or Δ*plcB* strain. (B) Gray value analysis of PCCA expression levels in HeLa cells. (C) RT-qPCR analysis of *pcca* transcript levels in HeLa cells after infection with the different strains. (D) Western blotting analysis of mitochondrial PCCA expression after infection with the different strains. (E) Gray value analysis of mitochondrial PCCA expression levels. Data are expressed as the mean ± SD ($n = 3$). ns means no significant differences.

Mitochondria are essential organelles in eukaryotic cells because of their critical roles in energy production, metabolic regulation, calcium signaling, apoptosis, and redox and innate immune signaling (24–26). Several mitochondrial metabolic enzymes, including succinate dehydrogenase and mitochondrial intermediate metabolites, have been reported to participate in bacterial killing (27, 28). PCCA is a mitochondrial carboxylase, whose primary function is to catalyze the carboxylation of propionyl-CoA to produce methylmalonyl-CoA. Since intracellular accumulation of propionyl-CoA can inhibit mitochondrial metabolism and reduce the synthesis of citrate, GTP, and ATP, PCCA is essential to supply TCA substrates and support TCA function (29). When PCCA is dysfunctional, it usually leads to a severe metabolic disorder demonstrating significant morbidity and mortality (30). Therefore, the availability of PCCA in mitochondria is a critical determinant of which cellular functions are modified. In the present study, the results revealed that the knockdown of PCCA facilitated bacterial survival, which might be related to the impaired LC3-associated phagocytosis (LAP)-mediated defense mechanism when PCCA was deficient, because phagosomes containing the bacterial pathogen *L. monocytogenes* can be targeted by LAP (31).

LAP is a host defense mechanism against invading pathogens that involves the formation of autophagosomes around the bacteria, leading to their degradation (18, 32–34). Previous study has suggested that macrophages target *Salmonella typhimurium* by LAP, where LC3 is directly recruited to phagocytosed bacteria in a manner dependent on the activation of reactive oxygen species (ROS) production in the *Salmonella*-containing compartment (35). LAP has also been evidenced to protect against *Aspergillus fumigatus* infection by activity of a Class III PI (3) K complex (36). Indeed, *L. monocytogenes*–derived LLO causes plasma membrane damage to induce an influx of extracellular Ca^{2+}/endoplasmic reticulum Ca^{2+} release and subsequent mitochondrial Ca^{2+}

($mtCa^{2+}$) uniporter-dependent mitochondrial uptake. Elevated $mtCa^{2+}$ levels augment pyruvate dehydrogenase (PDH) activity, leading to increased acetyl-CoA production in mitochondria. Accumulated acetyl-CoA in the mitochondria-phagosome connection area preferentially modifies the LAP-associated molecule Rubicon in a compartmentalized manner (37). The LAP-mediated defense mechanism is activated by Rubicon, an effector protein that acetylates acetyl-CoA. In the setting of PCCA deficiency, propionyl-CoA and propionate can inhibit pyruvate dehydrogenase, leading to decreased acetyl-CoA generation (38). As a result, acetyl-CoA-mediated acetylation of Rubicon is reduced, leading to decreased LAP formation (36, 37). This study revealed that PCCA abundance was negatively correlated with host cell infection by *L. monocytogenes*. These results suggested that *L. monocytogenes* might escape LAP-mediated bacterial killing due to reduced LAP when PCCA expression levels were decreased in host cells (Fig. 6). This finding provides new insights into the mechanisms by which *L. monocytogenes* evades host immune responses and highlights the importance of mitochondrial metabolism in the host defense against bacterial infections. Therefore, we suggest that PCCA plays an important role in the host defense against *L. monocytogenes* infection. This highlights the importance of mitochondrial metabolism in the immune response to bacterial infections and provides a new perspective for the development of novel therapeutic strategies to combat bacterial infections.

Due to their involvement in essential cellular processes, mitochondria represent attractive targets for viral and bacterial pathogens seeking to modulate host cellular processes to promote their survival and replication (11, 39–41). Several pathogenic bacteria have been shown to target mitochondrial dynamics and function to create favorable intracellular environments that promote their survival and persistence. For example, *Vibrio cholera*, which uses the type 3 secretion system effector VopE to activate mitochondrial GTPase activity and modulate mitochondrial trafficking, effectively blocks innate immune responses that presumably require mitochondria as signaling platforms (42). Mitochondria are highly dynamic organelles that constantly undergo fusion and fission. Mitochondrial dynamics during *L. monocytogenes* infection have been analyzed, and it has been shown that this infection profoundly alters mitochondrial dynamics by causing transient mitochondrial network fragmentation. The secreted pore-forming toxin LLO as a bacterial factor is mainly responsible for mitochondrial network disruption and mitochondrial function modulation (12). LLO can enhance *Listeria* entry into cells by inducing Ca^{2+} influx because Ca^{2+} influx probably represents the first bioenergetics insult to the cell, inducing mitochondrial fragmentation and depolarization as well as blocking mitochondrial movement (43–47). *L. monocytogenes* infection not only elicits transient mitochondrial fission and a drop in mitochondrion-dependent energy production through a mechanism requiring LLO, but also Mic10, a critical component of the mitochondrial contact site and cristae organizing system complex, for *L. monocytogenes*-induced mitochondrial network fragmentation (13, 48). In the present study, we found that *L. monocytogenes* PlcB protein interacts with the mitochondrial carboxylase PCCA, and the key structural domains and amino acid sites involved in the interactions between PlcB and mitochondrial PCCA were clearly identified. We also found that the knockdown of PCCA facilitated bacterial survival, possibly by impairing the LAP-mediated defense mechanism. However, the results revealed that PCCA expression in the mitochondria was not significantly affected by *L. monocytogenes* infection, highlighting the relevance of mitochondrial dynamics in *Listeria* infection. Pathogenic bacteria can target host cell organelles to control key cellular processes and promote their intracellular survival, growth, and persistence (11, 12, 14–16). Mitochondrial dynamics and function are closely linked, and bacterial manipulation of mitochondrial processes can create favorable intracellular environments for bacterial replication and survival. Therefore, it is possible that *L. monocytogenes* exploits the interaction between PlcB and PCCA to maintain stable PCCA expression and supply TCA substrates to create a favorable intracellular environment for its survival and growth. Further studies are needed to elucidate the precise mechanisms underlying this interaction and its role in *L. monocytogenes*.

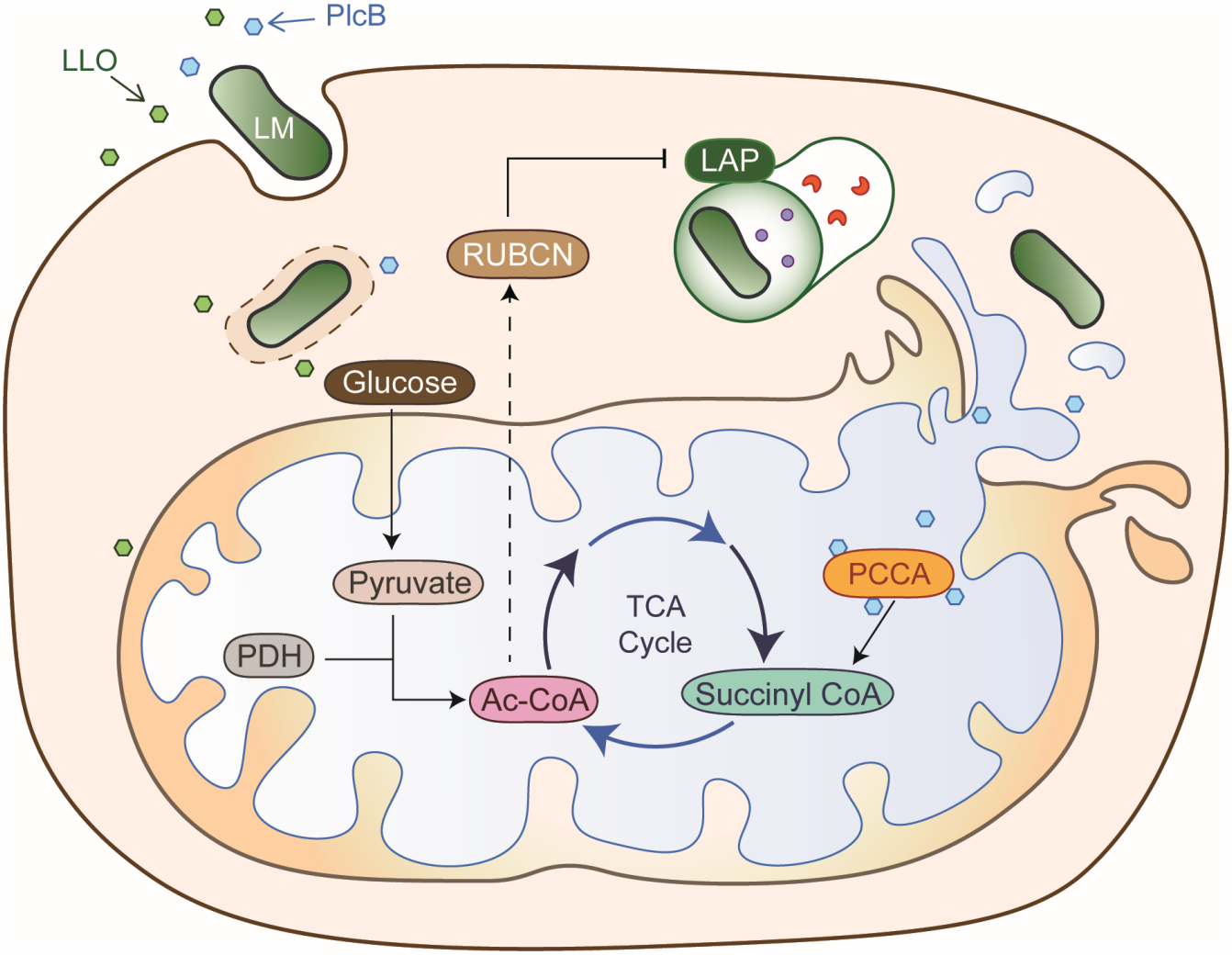

FIG 6 A proposed model describes the modulation of *L. monocytogenes* survival via the interaction between mitochondrial carboxylase PCCA and phospholipase PlcB. Under PCCA deficiency, the accumulation of propionyl-CoA and propionate can inhibit PDH activity, leading to a reduction in acetyl-CoA (Ac-CoA) generation. This subsequently results in the downregulation of Ac-CoA-mediated acetylation of Rubicon (RUBCN) and reduced LAP formation. Decreased LAP formation may reduce bacterial killing, allowing *L. monocytogenes* to evade this host defense mechanism. However, *L. monocytogenes* may leverage the interaction between PlcB and PCCA to maintain stable PCCA expression and provide TCA substrates to create a favorable intracellular environment for its survival and growth. The abbreviations used in the legend are LM for *L. monocytogenes,* and LLO for listeriolysin O.

In summary, this study sheds light on the previously unknown interaction between *L. monocytogenes* and host cell mitochondria, particularly the role of the mitochondrial carboxylase PCCA in bacterial survival. These findings suggest that upregulated PCCA levels can inhibit *L. monocytogenes* survival, highlighting the potential of PCCA as a target for the development of new antibacterial strategies. However, the interaction between PlcB and PCCA may allow *L. monocytogenes* to maintain stable PCCA expression during infection, which could be a key pro-survival strategy. Overall, this study provides new insights into the complex interplay between bacterial pathogens and host cell mitochondria, which could pave the way for new approaches for combating bacterial infections.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and culture conditions

L. monocytogenes strains, including wild-type EGD-e and its isogenic PlcB mutant Δ*plcB*, were used in this study. All *L. monocytogenes* strains were grown at 37°C in Brain Heart Infusion (Thermo Fisher Scientific, USA) supplemented with ampicillin (50 µg/mL), chloramphenicol (10 µg/mL), or kanamycin (50 µg/mL). *E. coli* DH5α cells were grown at 37°C in Luria-Bertani (LB) medium (Oxoid Ltd., United Kingdom) for transformation. HEK 293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS, GE Healthcare Hyclone), and grown at 37°C in a humidified 5% CO_2 atmosphere for protein expression. Primers used in this study are listed in Table S2.

Protein interaction assay using yeast two-hybrid test

In a previous study, we identified mitochondrial PCCA protein as a PlcB-interacting protein by screening a human cDNA library. To validate plasmid specificity in the yeast two-hybrid system and reduce false positives, we performed a back-up verification test. In the yeast two-hybrid assay, pGADT7-PCCA and pGBKT7-BD-PlcB were co-transfected into Y2HGold cells. The positive control group consisted of pGADT7-T and pGBKT7-53, whereas the negative control group consisted of pGADT7-T and pGBKT7-Lam. Y2HGold carrying the above plasmids was grown at 30°C in DDO, DDO/X, QDO, or QDO/X/A solid medium for 4 days, and colony growth was observed. If the colonies transfected with pGADT7-PCCA and pGBKT7-BD-PlcB plasmid can grow normally on QDO medium and turn blue in DDO/X and QDO/X/A medium, it indicates that the PCCA positive clonal back-up test is successful and interaction between PlcB and PCCA. DDO means Double dropout medium; DDO/X means DDO with additional addition X-α-Gal (1:500); QDO means Quadruple dropout medium; and QDO/X/A means QDO with additional addition X-α-Gal (1:500) and Aureobasidin A (AbA; 1:2,500).

Protein interaction assay using Co-IP

Primers with specific restriction sites (*EcoR* I and *Kpn* I) were designed from the known nucleotide sequences of *plc*B and *pcca*. The resulting PCR products were digested with EcoRI and KpnI and cloned into pCMV-N-Myc and pCMV-N-HA vectors digested with the same enzymes to generate the plasmids pCMV-N-Myc-$PlcB_{26\text{-}289}$ and pCMV-N-HA-$PCCA_{389\text{-}729}$. The expression plasmids were co-transfected into HEK293T cells to conduct an interaction assay using the collected cell-secreted proteins. To identify the key regions where PlcB specifically binds to PCCA, the PCCA protein sequence was truncated, and p-CMV plasmids carrying HA tags were constructed. The key structural domains and amino acid sites involved in the interactions between PlcB and mitochondrial PCCA were elucidated using Co-IP.

Protein colocalization assay using immunofluorescence

HEK293T cells were seeded in sixwell plates at a density of 2×10^5 cells/mL 24 h before the assay. Plasmid DNA encoding pCMV-N-Myc-PlcB or pCMV-N-HA-PCCA was mixed with JerPRIME (Polyplus, France) transfection reagent (1:2) and added to each well. After 48 h of transfection, cells were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.5% Triton X-100 for 5 min, and blocked with 1% bovine serum albumin (BSA) for 30 min. Primary antibodies were added, and the cells were incubated at 37°C in a blocking buffer for 1 h. After washing the cells three times with PBS, fluorophore-conjugated secondary antibodies or 4',6-diamidino-2-phenylindole (DAPI) dye was added for 1 h at 37°C. The coverslips were mounted onto microscope slides with mounting medium and imaged using a confocal microscope (Olympus, Japan). The primary antibodies used were Alexa Fluor 555 donkey anti-mouse IgG (H + L) (A-31570), Alexa Fluor 488 donkey anti-rabbit IgG (H + L; A-21206), β-tubulin

(D3U1W) mouse mAb, COX IV (4D11-B3-EB) mouse mAb, HA-Tag (C29F4) rabbit mAb, and Myc-Tag (9B11) mouse mAb.

Transient overexpression and gene knockdown of PCCA in HeLa cells

For transient overexpression experiments, HeLa cells were seeded in sixwell plates and transfected with 0.5 µg of pCMV-N-HA-PCCA plasmid DNA using jetPRIME (Polyplus, France) according to the manufacturer's instructions. Cells were transfected with an empty parental plasmid as a negative control (Ctrl). For transient gene knockdown experiments, HeLa cells were reverse transfected with small interfering RNA (siRNA) against PCCA (siRNA-si-PCCA) in 12-well plates using INTERFERin (Polyplus) transfection reagent. The cells were transfected with si-Ctrl as a negative control. siRNAs were produced by GenePharma Co., Ltd. (Shanghai, China).

Real-time quantitative PCR

The expression level of *pcca* was analyzed by extracting total RNA from HeLa cells using the Bacteria Total RNA Isolation Kit (TOYOBO, Japan) following the manufacturer's instructions. Specifically, cDNA was synthesized from 1 µg of total RNA using a Prime-Script RT reagent Kit with gDNA Eraser (Takara, Japan), followed by RT-qPCR using a LightCycler 480 Real Time System (Roche, Switzerland) with SYBR Green Real-time PCR Master Mix (Takara). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method after normalizing target gene values to those of the housekeeping gene *GAPDH*.

Analysis of PCCA expression using western blotting

To assess the knockdown or overexpression of PCCA during *L. monocytogenes* infection, PCCA protein levels were analyzed by western blotting. Briefly, the bacterial pellet was resuspended in 1 mL extraction solution (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, and pH 8.0), lysed using a homogenizer at 6,000 rpm for 30 s with intermittent cooling for 30 s, and then centrifuged at 12,000 × g for 15 min. The pellet was discarded, and the supernatant was retained as the whole-cell extract. HeLa cell mitochondria were extracted using a Mitochondrial Extraction Kit following the manufacturer's instructions (Solarbio, China). Protein samples were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated overnight with primary antibodies, including Myc-tag (9B11) mouse mAb, COX IV (4D11-B3-EB) mouse mAb, HA-Tag (C29F4) rabbit mAb, and goat anti-mouse or rabbit IgG (H + L) antibodies. Tubulin was used as an internal standard to normalize protein levels.

Intracellular growth of *L. monocytogenes* in HeLa cells

To investigate the role of PCCA in *L. monocytogenes* infection, intracellular growth assays were performed using HeLa cells with altered PCCA expression (overexpression or knockdown). Briefly, overnight-grown *L. monocytogenes* was washed and resuspended in PBS (pH 7.4). Monolayers of HeLa cells cultured in DMEM containing 10% FBS were infected with *L. monocytogenes* for 30 min at a multiplicity of infection (MOI) of 0.05 and incubated in DMEM containing gentamicin (50 µg/mL) for an additional 30 min to kill extracellular bacteria. Cells were treated with PBS as the control group. The infected cells were lysed with trypsin and distilled water 6 h post infection. The lysates were diluted and plated on brain heart infusion (BHI) agar plates to determine viable bacterial counts. The experiment was performed in triplicate, and a control group treated with PBS was included. Intracellular bacteria (% relative control) = (numbers of CFU counted in treatment group)/(numbers of CFU counted in control group) × 100%.

Statistical analysis

All data are presented as mean ± SD. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Duncan's test. Relative gene expression was

compared between the two groups using *t* tests. Differences were considered statistically significant at $P < 0.05$.

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DATA AVAILABILITY

Data used for analyses presented in this paper are available in the supplemental material.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Tables S1 and S2 (AEM02135-23-s0001.docx). Table S1, PCCA eukaryotic expression plasmids; Table S2, primers used in this study.

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