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Cloning, expression, and enzymatic activity of *Acinetobacter baumannii* and *Klebsiella pneumoniae* acetyl-coenzyme A carboxylases

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ABSTRACT

Pathogenic Gram-negative bacteria are a major public health concern because they are causative agents of life-threatening hospital-acquired infections. Due to the increasing rates of resistance to available antibiotics, there is an urgent need to develop new drugs. Acetyl-coenzyme A carboxylase (ACCase) is a promising target for the development of novel antibiotics. We describe here the expression, purification, and enzymatic activity of recombinant ACCases from two clinically relevant Gram-negative pathogens, *Acinetobacter baumannii* and *Klebsiella pneumoniae*. Recombinant ACCase subunits (AccAD, AccB, and AccC) were expressed and purified, and the holoenzymes were reconstituted. ACCase enzyme activity was monitored by direct detection of malonyl-coenzyme A (malonyl-CoA) formation by liquid chromatography tandem mass spectrometry (LC–MS/MS). Steady-state kinetics experiments showed similar k_{cat} and K_M values for both enzymes. In addition, similar IC_{50} values were observed for inhibition of both enzymes by a previously reported ACCase inhibitor. To provide a higher throughput assay suitable for inhibitor screening, we developed and validated a luminescence-based ACCase assay that monitors ATP depletion. Finally, we established an enzyme activity assay for the isolated AccAD (carboxyltransferase) subunit, which is useful for determining whether novel ACCase inhibitors inhibit the biotin carboxylase or carboxyltransferase site of ACCase. The methods described here could be applied toward the identification and characterization of novel inhibitors.

Gram-negative bacterial infections are a major public health concern because they are one of the most prevalent types of serious hospital-acquired infections [1–3]. They are responsible for a variety of bacteremias, with ventilator-associated pneumonia and bloodstream infections most frequently associated with poor outcomes [1–7]. Treatment of Gram-negative bacterial infections is remarkably challenging because these organisms have developed multiple strategies to neutralize antibacterials, including the outer membrane permeability barrier, the expression of drug efflux mechanisms, and continuous genetic adaptations [8]. During recent years, the emergence and increasing prevalence of multidrug-resistant (MDR)[2] and pandrug-resistant (PDR) Gram-negative bacteria have rendered current antibiotic therapies less effective. As a result, there is a critical need for development of new antibiotics active against MDR and PDR Gram-negative pathogens [9,10].

The enzyme acetyl-coenzyme A carboxylase (ACCase) has emerged as an attractive target for novel antibiotics. Bacterial ACCase is a multisubunit complex composed of three subunits: biotin carboxylase (AccC or BC), biotin carboxyl carrier protein (AccB or BCCP), and carboxyltransferase (AccA/AccD, AccAD, or CT) [11]. The holoenzyme catalyzes the conversion of acetyl-coenzyme A (acetyl-CoA) to malonyl-CoA in a two-step reaction. In the first step, the biotin carboxylase subunit (AccC) catalyzes the ATP-dependent carboxylation of a biotin group attached to AccB. In the second step, the carboxyltransferase subunit (AccAD) transfers the carboxyl group from carboxybiotin to acetyl-CoA, generating the product malonyl-CoA [12–15] (Fig. 1A). ACCase-catalyzed malonyl-CoA formation is the first-committed and rate-limiting step in the type II fatty acid biosynthesis pathway. Bacterial fatty acid synthesis is an essential pathway for bacterial growth and an established target for antimicrobial drugs, including isoniazid and triclosan [16–18]. The essentiality of the ACCase subunits has been demonstrated using genetic approaches [19–22]. ACCase subunits are highly conserved across Gram-positive and

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[2] *Abbreviations used:* MDR, multidrug-resistant; PDR, pandrug-resistant; ACCase, acetyl-coenzyme A carboxylase; BC (or AccC), biotin carboxylase; BCCP (or AccB), biotin carboxyl carrier protein; CT (or AccA/AccD or AccAD), carboxyltransferase; CoA, coenzyme A; LC–MS/MS, liquid chromatography tandem mass spectrometry; CPM, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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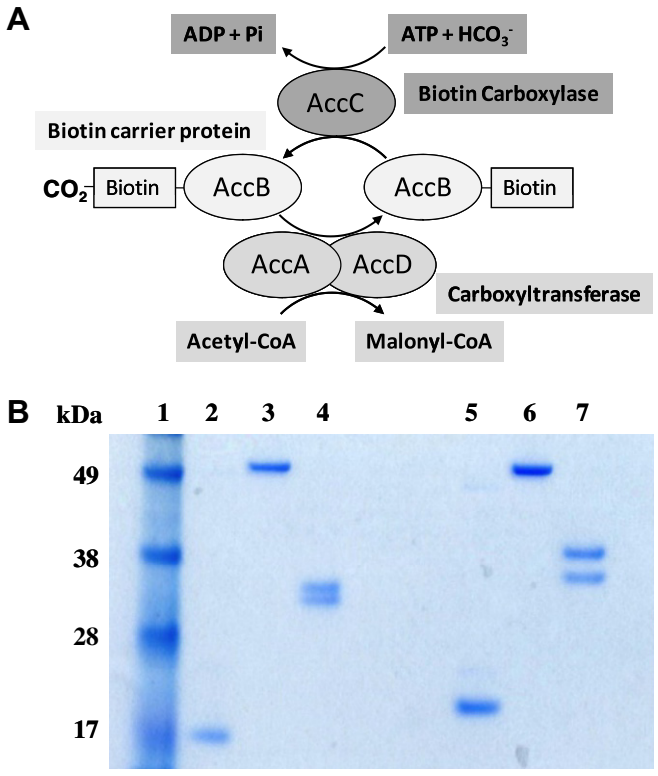

Fig.1. (A) Schematic diagram representing bacterial ACCase and the two-step reaction required for malonyl-CoA formation. (B) SDS–PAGE of ACCase subunits from *A. baumannii* and *K. pneumoniae.* Purified proteins (0.4 μg) were applied to NuPAGE 4 to 12% precast Bis–Tris gels, and bands were visualized using Gel Code Blue staining. Lane 1: SeeBlue Plus2 protein marker; lane 2: A. baumannii AccB; lane 3: *A. baumannii* AccC; lane 4: *A. baumannii* AccAD; lane 5: *K. pneumoniae* AccB; lane 6: *K. pneumoniae* AccC; lane 7: *K. pneumoniae* AccAD.

Gram-negative bacteria, suggesting the potential to identify ACCase inhibitors with broad-spectrum antibacterial activity. Inhibitors that target either the CT or BC domain of ACCase have been shown to have antibiotic activity both in vitro and in vivo [23–26].

Because ACCase is a promising target for novel antibacterials, understanding its biochemical characteristics is of utmost importance. In this study, we report the cloning, expression, purification, reconstitution, and enzyme activity characterization of *Acinetobacter baumannii* and *Klebsiella pneumoniae* ACCases. *A. baumannii* and *K. pneumoniae* ACCases were selected for this study because they are prominent among Gram-negative bacterial species responsible for drug-resistant hospital-acquired infections [2,27,28]. The enzyme activity of the holoenzymes was determined either by measuring malonyl-CoA formation using a liquid chromatography tandem mass spectrometry (LC–MS/MS) assay or by measuring ATP depletion using a Kinase-Glo assay. Finally, we performed inhibition studies using a previously reported pseudopeptide pyrrolidinedione inhibitor (AccAD inhibitor) with both holo-ACCase and the isolated AccAD subunit.

Materials and methods

Materials and reagents

Genomic DNA samples from *A. baumannii* strain 5377 and *K. pneumoniae* strain MGH78578 were obtained from American Type Culture Collection (ATCC). Pseudopeptide pyrrolidinedione inhibitor #7 [24] was kindly provided by Srinivasa Rao (Novartis Institute of Tropical Diseases, Singapore). Restriction enzymes BamHI, NotI, NcoI, NdeI, and SacI, Quick Ligation Kit, and Phusion Hot Start DNA Polymerase Kit were purchased from New England Biolabs. *Escherichia coli* strain BL21(DE3) and expression plasmids pET28a+ and pET42b were purchased from Novagen. QuikChange II XL Site-Directed Mutagenesis Kit was obtained from Agilent Technologies. Gel Code Blue was obtained from Pierce. Subcloning efficiency *E. coli* DH5α, NuPAGE 4–12% precast Bis–Tris gels, SeeBlue Plus2 protein marker, 10-mM dNTP mixture, and 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) were obtained from Invitrogen. QIAprep Spin Miniprep Kit and MinElute Gel Extraction Kit were purchased from Qiagen. Acetyl-CoA trilithium salt, malonyl-CoA lithium salt, [3-^{13}C]malonyl-CoA lithium salt, ATP disodium salt hydrate, D-biotin, 0.5 M ethylenediaminetetraacetic acid (EDTA) solution, citrate synthase, oxaloacetic acid, and biocytin were obtained from Sigma. Kinase-Glo Plus was purchased from Promega.

Cloning of ACCase subunits and biotin ligase from A. baumannii and K. pneumoniae

The construction of *A. baumannii* and *K. pneumoniae* AccAD bicistronic coexpression vectors was performed using the overlap extension protocol [29]. Briefly, AccA and AccD sequences were amplified from genomic DNA by polymerase chain reaction (PCR) using the following oligonucleotides: *A. baumannii* AccA fw primer (5′-AAAA GGATCC ATG AAA AAA GCT ACT CAG TCC AAA GC-3′) and *A. baumannii* AccAD bicistronic rev primer (5′-CC TGA TTT CAC TTC TTG ATT CAT ***ctcgagttctcc*** TTA AGA CGC CAT GCC TAA ATT TTC-3′); *A. baumannii* AccAD bicistronic fw primer (5′-GAA AAT TTA GGC ATG GCG TCT TAA ***ggagaactcgag*** ATG AAT CAA GAA GTG AAA TCA GG-3′) and *A. baumannii* AccD rev primer (5′-TTTT GCGGCCGC TCA AGG TAA GTT CAT CAA TTT TGA TAC-3′); *K. pneumoniae* AccA fw primer (5′-AAAA GGATCC ATG AGT CTG AAT TTC CTA GAT TTT GAA C-3′) and *K. pneumoniae* AccAD bicistronic rev primer (5′-CT TTT AAT TCG TTC AAT CCA GCT CAT ***ctcgagttctcc*** TTA AGC GTA ACC GTA GCT CAT C-3′); *K. pneumoniae* AccAD bicistronic fw primer (5′-G ATG AGC TAC GGT TAC GCT TAA ***ggagaactcgag*** ATG AGC TGG ATT GAA CGA ATT AAA AG-3′) and *K. pneumoniae* AccD rev primer (5′-TTTT GCGGCCGC TCA GGC CTC AGG TTC CTG ATC C-3′). The sequences in bold italic lowercase correspond to the ribosomal entry site [30,31], and the underlined italic uppercase sequences correspond to BamHI and NotI restriction sites.

The following oligonucleotides were used to generate *A. baumannii* and *K. pneumoniae* AccC, AccB, and BirA DNAs: *A. baumannii* AccC fw primer (5′-AAAA GGATCC ATG TTG CAA AAA GTT TTA ATT GCA AAC C 3′) and *A. baumannii* AccC rev primer (5′-TTTT GCGGCCGC TTA AGC AGT TTC AGC TTT CTT TTC TTC TTC-3′); *A. baumannii* AccB fw primer (5′-AAAA CCATGG ***gg*** ATG GAT ATC CGC AAA ATT AAG AAA CTC-3′) and *A. baumannii* AccB rev primer (5′-TTTT GAGCTC CGC GCG ATA ACG GAA TAA AGG-3′); *A. baumannii* BirA fw primer (5′-AAAA CATATG GAT TTA GAG ACT CGC CAA CTA C-3′) and *A. baumannii* BirA rev primer (5′-TTTT GAGCTC TTA CTG AGT GGT TTG TGG TCT TAG-3′); *K. pneumoniae* AccC fw primer (5′-AAAA GGATCC ATG CTG GAT AAA ATT GTC ATC GCT AAC-3′) and *K. pneumoniae* AccC rev primer (5′-TTTT GCGGCCGC TTA TTT TTC CTG CAA GCC GAG TTT TTT C-3′); *K. pneumoniae* AccB fw primer (5′-AAAA CCATGG ***gg*** ATG GAT ATT CGT AAG ATT AAA AAA CTG-3′) and *K. pneumoniae* AccB rev primer (5′-TTTT GAGCTC CTC GAT AAC TAC CAG CGG C-3′); *K. pneumoniae* BirA fw primer (5′-AAAA CATATG AAA GAC CAT ACT ATC CCT TTA AC-3′) and *K. pneumoniae* BirA rev primer (5′-TTTT GAGCTC TTA TTC TGC ACT GCG CAA TGA TAT TTC-3′). The bold italic lowercase guanine from AccB forward primers was added to correct the reading frame disrupted by the NcoI restriction site. The underlined italic uppercase sequences correspond to BamHI and NotI (AccC), NcoI and SacI (AccB), and NdeI and SacI (BirA) restriction sites.

Phusion Hot Start DNA Polymerase Kit was used for PCR reactions according to the manufacturer's protocol (New England Biolabs). Briefly, the DNA sequences were amplified in 50-μl reactions (1× Phusion GC Buffer, 10 mM dNTP mixture, 2 μM primer mix, and Phusion Hot Start DNA Polymerase). The number of cycles used was 30, and the following cycling parameters were used: denaturing step for 10 s at 98 °C, annealing step for 30 s at 50 °C, and extension step for 2 min at 72 °C, followed by a final extension step for 7 min at 72 °C. The PCR products were gel purified using the MinElute Gel Extraction Kit. Fusion of AccA and AccD sequences was achieved in a second PCR round, where the PCR products were combined and amplified using AccA fw and AccD rev primers. The PCR products were gel purified, digested with their corresponding restriction enzymes, and subcloned in a 3:1 molar ratio into digested pET28a+ (AccB, AccC, and AccAD) or pET42b (BirA) using the Quick Ligation Kit according to the manufacturer's instructions (New England Biolabs). The ligation products were used to transform subcloning efficiency *E. coli* DH5α, and plasmids were purified using the QIAprep Spin Miniprep Kit and confirmed by DNA sequencing. Finally, the QuikChange II XL Site-Directed Mutagenesis Kit was used to correct a frame shift affecting the C-terminal 6×His tag of AccB-pET28a+. The following primers were used: primer A fw (5′-CGT CGA CAA GCT TCG GCC GCA CTC GAG-3′) and primer B rev (5′-CTC GAG TGC GGC CGA AGC TTG TCG ACG-3′).

Expression and purification of recombinant ACCase subunits

The plasmids 6×His-AccAD-pET28a+, 6×His-AccC-pET28a+, AccB-6×His-pET28a+, and BirA-pET42b were used to transform *E. coli* BL21(DE3). Single colony-forming units were used to inoculate 5 ml of Luria–Bertani (LB) medium supplemented with 50 μg/ml kanamycin and incubated at 37 °C overnight. The medium volume was successively increased to 1 L, and the cell density was adjusted to $OD_{600} \sim 0.4$. Protein expression was initiated by the addition of 1 mM isopropyl-3-D-thiogalactoside (IPTG), and the cell culture was maintained at 30 °C for 16 h. The cells were then harvested by centrifugation at 4 °C, and the pellets from 6×His-AccAD- and 6×His-AccC-overexpressing cell cultures were resuspended in 50 ml of binding buffer (50 mM Tris–HCl [pH 8.0], 300 mM NaCl, 10% [v/v] glycerol, and 20 mM imidazole) for sonication. Cell pellets containing AccB-6×His and BirA were resuspended in biotin ligase buffer (see below). The supernatant was recovered by centrifugation at 17,000*g* for 45 min at 4 °C, and the purification step was performed using an AKTA fast protein liquid chromatography (FPLC) device (Amersham Biosciences) with a HisTrap 5-ml HP column (GE Life Sciences). The supernatant was applied to the column at 1.5 ml/min, and the column was washed with two column volumes of binding buffer. The enzyme was eluted from the column by linearly increasing the imidazole concentration from 20 to 500 mM, and fractions containing the protein of interest were pooled and evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). To remove imidazole, the pooled fractions were buffer-exchanged into storage buffer (50 mM Tris–HCl [pH 8.0], 300 mM NaCl, and 10% [v/v] glycerol) using Amicon Ultra centrifugal filter devices (Millipore). The purified protein was flash-frozen and stored at −80 °C.

In vitro biotinylation of AccB

The biotinylation protocol used here was adapted from previously reported methods [32,33]. Briefly, AccB-6×His- and BirA-overexpressing cell pellets were resuspended in biotin ligase buffer (40 mM sodium phosphate [pH 8.0], 1.5 mM $MgCl_2$, 5% [v/v] glycerol, 1 mM dithiothreitol [DTT], and 1 mM D-biotin) and sonicated. The supernatant was recovered by centrifugation at 17,000*g* for 45 min at 4 °C and filtered. AccB-6×His and BirA cell-free extracts were then combined, and ATP was added to a final concentration of 10 mM. The biotinylation reaction was incubated with constant shaking, initially at 37 °C for 1 h and then overnight at 4 °C. AccB-6×His was then purified by immobilized metal affinity chromatography as above, and biotinylation was evaluated by intact mass measurement. The ACCase subunits were then in vitro reconstituted and stored at −80 °C.

LC–MS/MS-based assay for monitoring ACCase carboxyltransferase activity

The assay conditions described by Soriano and coworkers [33] were adapted to an LC–MS/MS format. Malonyl-CoA formation was monitored in activity buffer (98 nM ACCase in 100 mM Hepes [pH 8.0], 5 mM HCO_3^-, 0.5 mM $MgCl_2$, 10% [v/v] glycerol, 40 μM acetyl-CoA, 10 μM ATP, and 1 mM DTT). Reactions were performed in a 40-μl final volume in V96 MicroWell plates (Nunc). Enzyme reactions were initiated by the addition of ATP, acetyl-CoA, or the holoenzyme and were incubated at room temperature for 20 min (*A. baumannii* ACCase) or 5 min (*K. pneumoniae* ACCase). Reactions were stopped by adding 10 μl of 10% (v/v) acetic acid containing 13.5 μM [3-^{13}C]malonyl-CoA as an internal standard. A 5-μl sample of each quenched reaction mixture was then transferred to a 384-well plate containing 45 μl of reaction dilution buffer (100 mM Hepes [pH 8.0], 5 mM HCO_3^-, 0.5 mM $MgCl_2$, 10% [v/v] glycerol, and 1 mM DTT). The diluted samples were then stored at −80 °C prior to LC–MS/MS analysis. Malonyl-CoA (Sigma) standard curves were prepared for all experiments.

The LC–MS system consisted of a PAL HTS Autosampler (Leap Technologies) equipped with a thermostatted cold stack for plate storage. Mobile phase was delivered using an Agilent G1312A Binary Pump (Agilent Technologies). A Micromass Quattro Micro triple quadrupole mass spectrometer (Waters) was used for high-sensitivity detection. The high-performance liquid chromatography (HPLC) column used was a C18 Synergi Max RP column (100 × 2.0 mm, 4 μm, Phenomenex). A 5-μl sample loop was overfilled 3× with the reaction mixture prior to analysis. Sample plates were stored at 10 °C in the PAL's thermostatted cold stack. The HPLC method was carried out at a flow rate of 0.5 ml/min at room temperature. Mobile phase A consisted of 5 mM dihexylammonium acetate (DHAA) and 10 mM ammonium acetate and was adjusted to pH 9.0 with ammonium hydroxide. Mobile phase B was unmodified acetonitrile. The gradient consisted of an initial 0.25-min hold at 5% B followed by a 5.75-min gradient from 5% B to 50% B. From 6 to 7.5 min, the mobile phase was held at 50% B. At 7.51 min, the mobile phase was returned to initial conditions and held until 9 min.

A positive electrospray ionization (ESI) multiple reaction monitoring (MRM) MS/MS method was used for analyte detection. For acetyl-CoA, the transition from 810.45 to 303.2 was monitored. For malonyl-CoA, the transition from 854.5 to 347.1 was monitored. For [3-^{13}C]malonyl-CoA, the transition from 857.6 to 350.2 was monitored. Each transition was monitored for 0.25 s with a 0.05-s interscan time; the total scan time was 0.9 s. For the first 5 min of each analytical run and from 7.5 to 9 min, the mobile phase was diverted to waste through the mass spectrometer's onboard divert valve. From 5 to 7.5 min, the entire 0.5-ml/min flow rate was directed into the ion source.

Luminescence-based assay for monitoring ACCase ATPase activity

ATPase activity was monitored using Kinase-Glo Plus according to the manufacturer's instructions (Promega). All assays were performed in V96 MicroWell plates. The assay conditions were similar to the method established for the LC–MS/MS assay above.

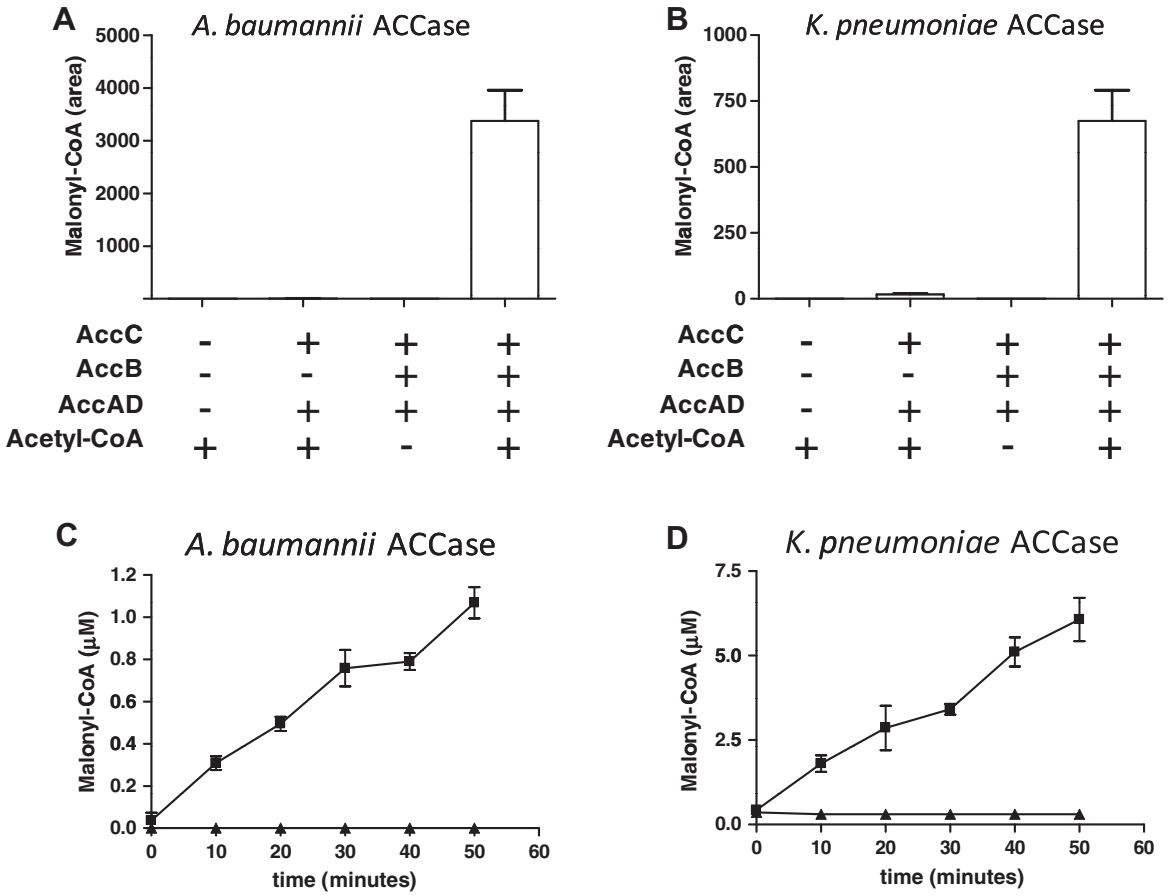

Fig.2. ACCases catalyze malonyl-CoA formation in vitro. (A, B) *A. baumannii* (A) and *K. pneumoniae* (B) ACCase activities were confirmed by LC–MS/MS. Enzymes were incubated for 3 h at room temperature in 100 mM Hepes (pH 8.0), 5 mM HCO_3^-, 0.5 mM $MgCl_2$, 10% (v/v) glycerol, 10 μM ATP, and 1 mM DTT. Product formation was not detected in the absence of AccB. (C, D) Time course assays for malonyl-CoA formation by *A. baumannii* (C) and *K. pneumoniae* (D) ACCases performed in the presence (■) or absence (▲) of acetyl-CoA. Here 40-μl reactions were quenched by acetic acid and malonyl-CoA was measured at the indicated time points. Results are representative of two independent experiments performed in triplicate.

Briefly, approximately 780 nM ACCase was incubated at room temperature in activity buffer for 20 and 5 min for *A. baumannii* and *K. pneumoniae* ACCases, respectively. Reactions were initiated by the addition of holoenzyme or substrates, and 5 mM EDTA (10 μl) was added to quench ACCase activity. Then 5 μl of each reaction was transferred to a Costar 96-well flat-bottom white polystyrene plate (Corning) containing 45 μl of reaction dilution buffer. Then 50 μl of Kinase-Glo Plus was added to each well and incubated at room temperature for 10 min. Luminescence was monitored using a Chemiluminescence Imaging Plate Reader (Molecular Devices). A malonyl-CoA standard curve was generated for all experiments.

Indirect fluorometric assay for AccAD activity

The protocol used was modified from Santoro and coworkers [34]. The reaction buffer used for the reverse carboxyltransferase assay contained ⩽20 nM recombinant 6×His-AccAD domain in 100 mM sodium phosphate (pH 7.0), 11.7 mM biocytin, 18 mM oxaloacetic acid, and 3.8 U of citrate synthase. All reactions were performed in 384-well plates. CPM stocks (10 mM) were prepared in dimethyl sulfoxide (DMSO), and malonyl-CoA stocks (5 mM) were prepared in 100 mM sodium phosphate (pH 7.0). For the enzyme activity assay, recombinant 6×His-AccAD domain in 47 μl of reaction buffer was dispensed, and the reaction was initiated by the addition of 2 μl of malonyl-CoA followed by 1 μl of CPM. Malonyl-CoA and CPM final concentrations were 200 and 100 μM, respectively. The fluorescence increase was monitored continuously at λex = 384 nm and λem = 470 nm in kinetic mode for 1 h at room temperature using a SpectraMax Gemini XS spectrofluorimeter (Molecular Devices).

Enzyme inhibition

Enzyme inhibition experiments using the pseudopeptide pyrrolidinedione inhibitor #7 [24] (referred to herein as AccAD inhibitor) were performed at room temperature. For the LC–MS/MS- and Kinase-Glo Plus-based experiments, 1 μl of serially diluted AccAD inhibitor or DMSO was dispensed first, followed by the addition of ACCase in activity buffer without acetyl-CoA. The reactions were incubated for 10 min at room temperature, followed by the addition of 1 μl acetyl-CoA. For the indirect fluorometric assays, 1 μl of AccAD inhibitor or DMSO was dispensed to the wells, followed by the addition of 46 μl recombinant 6×His-AccAD domain in reaction buffer. After incubation for 20 min at room temperature, the reaction was initiated by the addition of 2 μl malonyl-CoA and 1 μl CPM. As a control, the coupling enzyme reaction was incubated under the same conditions in the absence of recombinant 6×His-AccAD, and 50 μM acetyl-CoA was used as the substrate.

Data analysis and statistics

Data were analyzed using GraphPad Prism (GraphPad Software) and Excel (Microsoft). The steady-state rate constants k_{cat}, K_M, and apparent K_M for the substrates acetyl-CoA and ATP were obtained

by analyzing the initial velocities as a function of substrate concentration using nonlinear regression fit to the Michaelis–Menten equation. For K_M determinations, the fixed substrate concentration for both acetyl-CoA and ATP was 200 μM. IC50 values for the AccAD inhibitor were determined by using a nonlinear regression fit to a one-site competitive binding equation.

Results

Cloning, expression, and purification of recombinant A. baumannii and K. pneumoniae ACCase subunits

Because ACCase is essential for bacterial growth and survival [22] and, therefore, is an attractive target for the development of specific inhibitors with antibacterial activity, we set to generate and characterize recombinant *A. baumannii* and *K. pneumoniae* holoenzymes. Because the carboxyltransferase domain subunits AccA and AccD are distantly located in *Pseudomonas aeruginosa* and other bacterial genomes [35], we generated a bicistronic AccA/AccD coexpression vector to enable expression and purification of the AccAD complex. A similar strategy has been used for the expression of the *E. coli* ACCase carboxyltransferase domain and *E. coli* aspartate transcarbamylase [30,31]. Bicistronic coexpression vectors were generated by inserting a 12-bp sequence corresponding to the internal ribosomal entry site 5′-ggagaactcgag-3′ [31] between the AccA and AccD coding sequences. The PCR-amplified fragments were subcloned into pET28a+ in frame with the affinity tag. The remaining subunits AccB and AccC were generated using standard procedures and subcloned into pET28a+.

We then performed heterologous expression of the ACCase subunits from *A. baumannii* and *K. pneumoniae* in *E. coli* BL21(DE3). All subunits were purified to near homogeneity from the soluble fraction using immobilized metal affinity chromatography after overnight overexpression in *E. coli* (Fig. 1B). It is noteworthy that the use of a bicistronic coexpression vector caused a slightly higher expression of 6×His-AccA compared with AccD, suggesting a bias toward the expression of the sequence upstream from the internal ribosomal entry site [36]. For all subunits, the apparent molecular weight based on SDS–PAGE gel migration matches the calculated molecular weight.

Because the AccB subunit requires biotinylation for the holoenzyme to be catalytically active [33], biotin ligase genes (BirA) from *A. baumannii* and *K. pneumoniae* were cloned from bacterial genomic DNA and untagged BirA-pET42b constructs were generated. In vitro biotinylation was induced by the addition of ATP and D-biotin to a mixture of cell-free extracts from AccB-6×His- and BirA-overexpressing cultures. To assess the requirement for and efficiency of this biotinylation protocol, AccB-6×His was also prepared without coincubation with BirA extract, ATP, and D-biotin. Based on their protein sequences, the expected masses for *A. baumannii* apo- and biotinylated AccB-6×His are 16,816.6 and 17,042.9 Da, respectively. For *K. pneumoniae* AccB-6×His, the apoprotein has an expected molecular weight of 18,542.3, whereas biotin-AccB-6×His is 18,786.6 Da. Intact protein mass spectrometry analyses indicated that more than 90% biotinylation was achieved after overnight incubation with BirA extract, ATP, and D-biotin (data not shown). In contrast, the AccB-6×His control samples that were not subjected to the biotinylation protocol had only approximately 5–15% biotinylation. This low level of biotinylation in the control samples was presumably due to endogenous *E. coli* BirA activity.

Holo-ACCase was prepared by in vitro reconstitution of AccAD, biotin-AccB, and AccC subunits. The subunits were combined in a ratio of 1:3:1.9 for AccC/AccAD/AccB [33]. Approximately 3 μM AccAD, 1 μM AccC, and 1.9 μM AccB subunits were used to generate *A. baumannii* ACCase, whereas 2.3 μM AccAD, 760 nM AccC, and 1.4 μM AccB were used to generate *K. pneumoniae* ACCase.

Table 1
Steady-state constants for *A. baumannii* and *K. pneumoniae* ACCases.

	k_{cat} (s^{-1})	K_M, acetyl-CoA (μM)	K_M, ATP (μM)
A. baumannii ACCase	11.6 ± 4.2	8.8 ± 0.4	103.7 ± 32.3
K. pneumoniae ACCase	30.1 ± 6.3	48.7 ± 14.6	83.1 ± 5.5

Note: Kinetic parameters were obtained using nonlinear regression fit to the Michaelis–Menten equation. The substrates acetyl-CoA and ATP were used at 200 μM for K_M determinations. Values correspond to averages of two independent experiments performed in triplicate with standard deviations indicated.

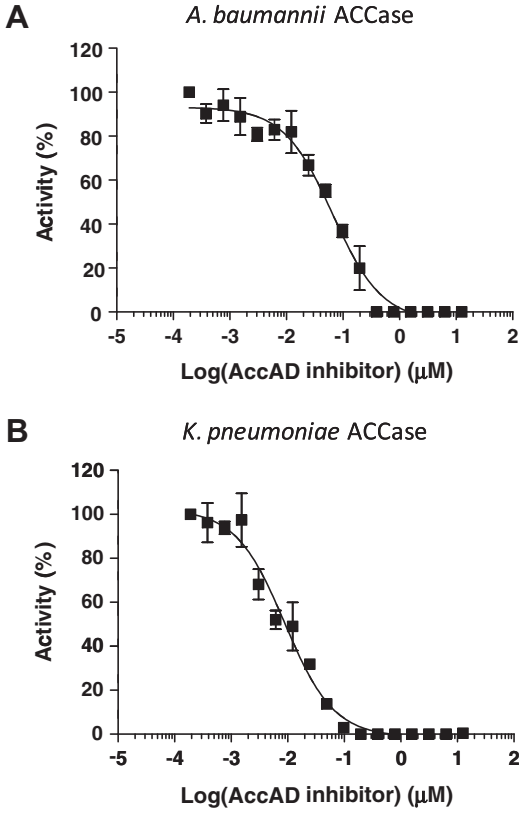

Fig.3. Dose–response assays. Holoenzymes were incubated with varying concentrations of the pseudopeptide pyrrolidinedione inhibitor. The IC_{50} values were 33 and 8 nM for *A. baumannii* (A) and *K. pneumoniae* (B) ACCases, respectively. Endpoint times for the reactions were 20 and 5 min for *A. baumannii* and *K. pneumoniae* ACCases, respectively, and reactions were terminated with the addition of acetic acid. Results correspond to two independent experiments performed in triplicate.

Recombinant ACCases catalyze the conversion of acetyl-CoA into malonyl-CoA

To assess whether the reconstituted ACCases were enzymatically active, we established an LC–MS/MS-based assay to directly monitor malonyl-CoA formation. *A. baumannii* and *K. pneumoniae* holoenzymes were incubated in the presence of acetyl-CoA and ATP for 3 h at room temperature. Malonyl-CoA formation was observed when all three subunits were present in the reaction buffer (Fig. 2A and B). In contrast, AccB removal eliminated ACCase activity, confirming the requirement [33] (Fig. 2A and B). No activity was observed when individual subunits were evaluated, indicating that no contaminating *E. coli* ACCase was present.

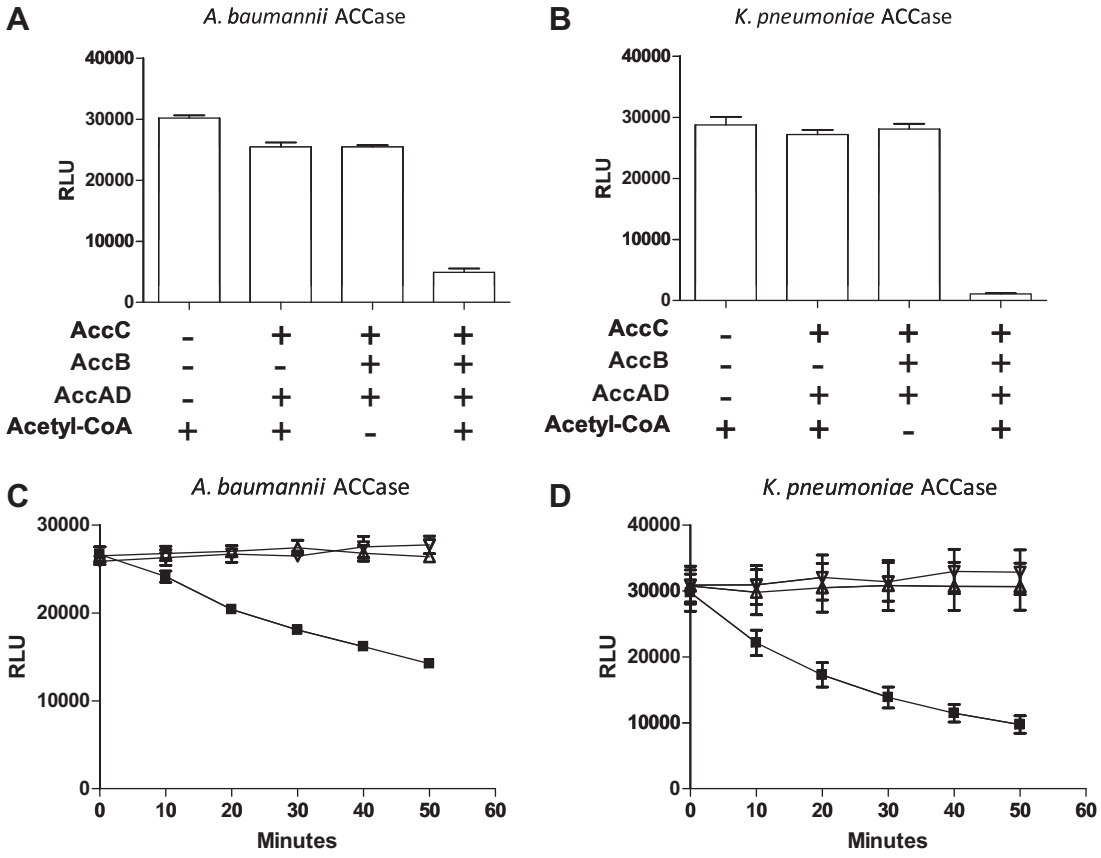

Fig.4. ACCase assays using Kinase-Glo Plus. (A,B) *A. baumannii* (A) and *K. pneumoniae* (B) ACCases were incubated in 100 mM Hepes (pH 8.0), 5 mM HCO_3^-, 0.5 mM $MgCl_2$, 10% (v/v) glycerol, 10 μM ATP, and 1 mM DTT for 3 h at room temperature. Reactions were quenched with EDTA, diluted, and incubated with Kinase-Glo Plus. Product formation was monitored as relative luminescence units (RLU). (C and D) Time course assays for ATP depletion by *A. baumannii* (C) and *K. pneumoniae* (D) holoenzymes. Activities were monitored for 50 min at room temperature in the presence (■) or absence (▽) of ACCase or the absence (△) of malonyl-CoA. Results are representative of three independent experiments.

Kinetic characterization of recombinant ACCases

Next, ACCase activity was evaluated in time course experiments. Both enzymes displayed linear product formation for up to 50 min at room temperature (Fig. 2C and D). For subsequent experiments, the reaction time and enzyme concentration were reduced to limit substrate consumption. Steady-state kinetic parameters k_{cat} and K_M were determined for both enzymes (Table 1). The kinetic constants were similar for both enzymes; the largest difference was a 4-fold difference between K_M values for acetyl-CoA. Finally, the holoenzymes were evaluated with respect to their susceptibility to inhibition by a reported pseudopeptide pyrrolidinedione inhibitor [24] that binds to and inhibits the carboxyltransferase (AccAD) domain. Dose–response assays were performed where the holoenzymes were incubated with serial dilutions of the inhibitor. The enzymatic activities of both enzymes were inhibited with apparent IC50 values of 33 and 8 nM for *A. baumannii* and *K. pneumoniae* ACCases, respectively (Fig. 3A and B). These IC50 values may underestimate the potency of these compounds because they are comparable to or less than the enzyme concentration. These results are in agreement with the high potency reported with this series of compounds for inhibition of *E. coli* ACCase [23,24].

Characterization of ATPase activity from A. baumannii and K. pneumoniae ACCases

Because ATP-dependent carboxylation of the biotin group precedes malonyl-CoA formation, we adapted an enzyme-coupled luminescence assay (Kinase-Glo Plus) to characterize the biotin carboxylase activity of both bacterial ACCases. Because the luminescence signal generated by the Kinase-Glo Plus reagent is proportional to the concentration of ATP, enzyme activity and corresponding ATP consumption is manifested as a decrease in the luminescence signal. In agreement with the results from the LC–MS/MS assay readout, both *A. baumannii* and *K. pneumoniae* ACCase enzymes were active in this assay (Fig. 4A and B) and displayed a continuous decrease in signal for 50 min (Fig. 4C and D). A holoenzyme concentration of at least 750 nM was necessary to observe activity of both the *A. baumannii* and *K. pneumoniae* enzymes with this Kinase-Glo Plus assay.

Next, we determined the apparent K_M values for acetyl-CoA. For these experiments, the ATP concentration was fixed at 100 μM, which is the maximum ATP concentration recommended for use with Kinase-Glo Plus. The K_M(app) values for both *A. baumannii* and *K. pneumoniae* holoenzymes were 24 and 48 μM, respectively (Fig. 5A and B). These K_M values are in good agreement with the results obtained using the LC–MS/MS readout.

The holoenzymes were completely inhibited at approximately 750 nM by the pseudopeptide pyrrolidinedione inhibitor [24] (Fig. 5C and D). Due to the high enzyme concentration used, it was not possible to determine a meaningful IC50 value.

Isolated carboxyltransferase subunit produces acetyl-CoA in vitro

The catalytic activity of isolated biotin carboxylase (AccC) and carboxyltransferase (AccAD) subunits can be monitored with appropriate assays [30,37]. In the absence of AccC and biotinylated

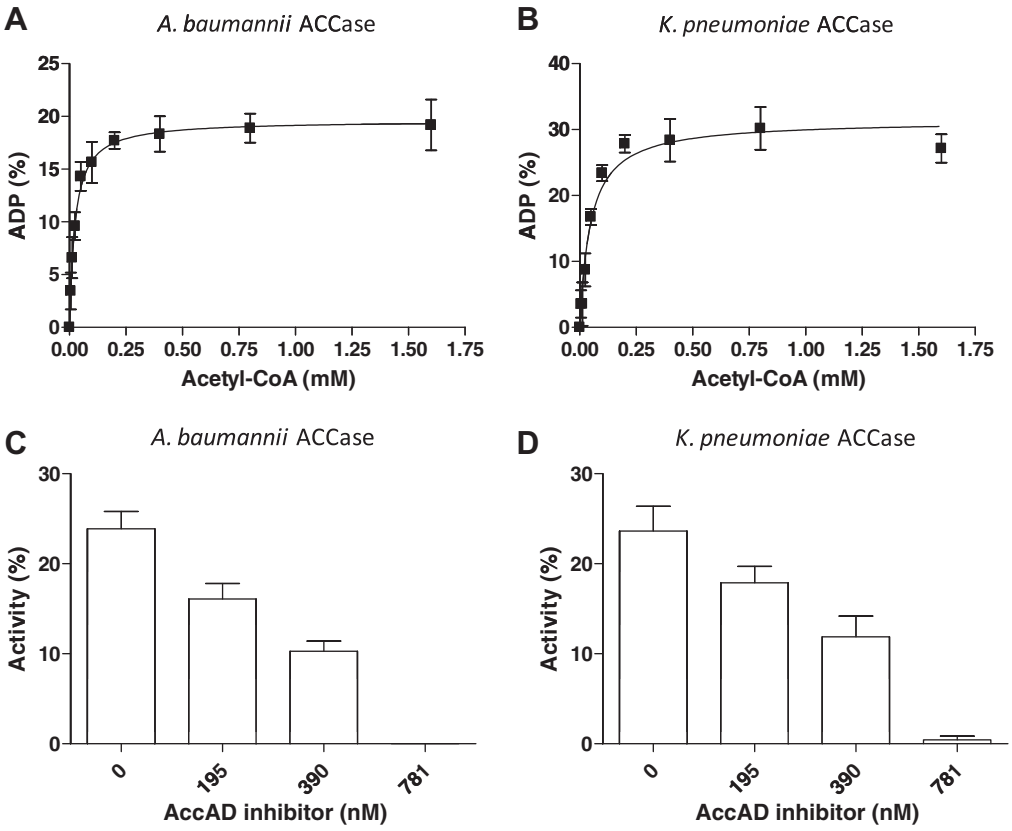

Fig.5. Biotin carboxylase activity characterization. (A,B) Varying concentrations of the substrate acetyl-CoA were incubated with *A. baumannii* (A) and *K. pneumoniae* (B) ACCases, and their respective K_M(app) values were 24 and 48 μM. (C,D) *A. baumannii* (C) and *K. pneumoniae* (D) ACCases were incubated with increasing concentrations of AccAD inhibitor. Complete inhibition was observed with 781 nM inhibitor. Endpoint times for the reactions were 20 and 5 min for *A. baumannii* and *K. pneumoniae* ACCases, respectively, and EDTA was used to quench reactions. Results correspond to three independent experiments.

AccB, recombinant AccAD activity can be monitored in the reserve orientation (i.e., conversion of malonyl-CoA to acetyl-CoA) provided that either free biotin or a biotin analog such as biocytin is used [30].

For the detection of bacterial carboxyltransferase activity in vitro, we adapted a reported enzyme-coupled assay to a fluorometric readout [34]. In this format, AccAD converts malonyl-CoA and biocytin to acetyl-CoA and carboxybiocytin. Acetyl-CoA is subsequently converted to CoA by citrate synthase in the presence of oxaloacetate. In the previously reported version of this assay, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) is used to detect the free thiol group of CoA. The reaction of DTNB with a free thiol group generates thionitrobenzoate, which is detected by its absorption at 412 nm [34]. We replaced DTNB with the thiol-reactive fluorogenic compound 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) (Fig. 6A). In the modified assay with CPM, fluorescence increase resulting from CoA formation was monitored continuously at λex = 384 nm and λem = 470 nm (Fig. 6B and C). We initially observed a rapid loss of signal due to an apparent incompatibility between CPM and the other reagents (data not shown). This was resolved by combining the enzyme and all reagents except the substrate malonyl-CoA and CPM first, followed by the sequential addition of malonyl-CoA and CPM. These changes resulted in a robust signal and the maintenance of assay linearity for up to 1 h at room temperature (Fig. 6B and C). CPM has been previously used to monitor CoA formation by the enzyme fatty acid synthase (FAS) [38].

Finally, the pseudopeptide pyrrolidinedione analog inhibitor was incubated with recombinant AccAD domains [24]. The apparent IC50 values for both AccAD subunits with this inhibitor were 17 and 8 nM for *A. baumannii* and *K. pneumoniae* carboxyltransferase domains, respectively (Fig. 7). These apparent IC50 values are similar to the results observed for the holoenzymes (Fig. 3A and B). Control experiments showed that the inhibitor does not interfere with the citrate synthase coupling enzyme.

Discussion

The shrinking antibacterial pipeline, together with the scarcity of drugs with Gram-negative spectrum and the rapid development of resistance, stresses the urgency to identify novel bacterial targets for the discovery of new antibiotics. Bacterial acetyl-CoA carboxylase is an attractive target for drug discovery due to the availability of inhibitors of both the AccC and AccAD subunits with demonstrated antibacterial activity in vitro and in vivo [23,24] and due to the demonstrated requirement for ACCase activity for bacterial growth and survival [19–22]. We prepared recombinant holo-ACCase from two major Gram-negative pathogens, *A. baumannii* and *K. pneumoniae*, and characterized their biochemical properties. Both of these bacterial species are important human pathogens. The number of *Acinetobacter*-related infections in intensive care unit (ICU) patients increased considerably between 1986 and 2003 [2]. Like *P. aeruginosa*, *A. baumannii* is an intrinsically drug-resistant pathogen and has the potential to develop resistance to other antibiotics [27]. Therefore, due to selective pressure from antimicrobials in hospital environments, *A. baumannii*-related infections may become more prevalent. *K. pneumoniae* is among the most common Gram-negative pathogens to cause bloodstream and urinary tract infections. Of particular concern is the increase in resistance to third-generation cephalosporins and

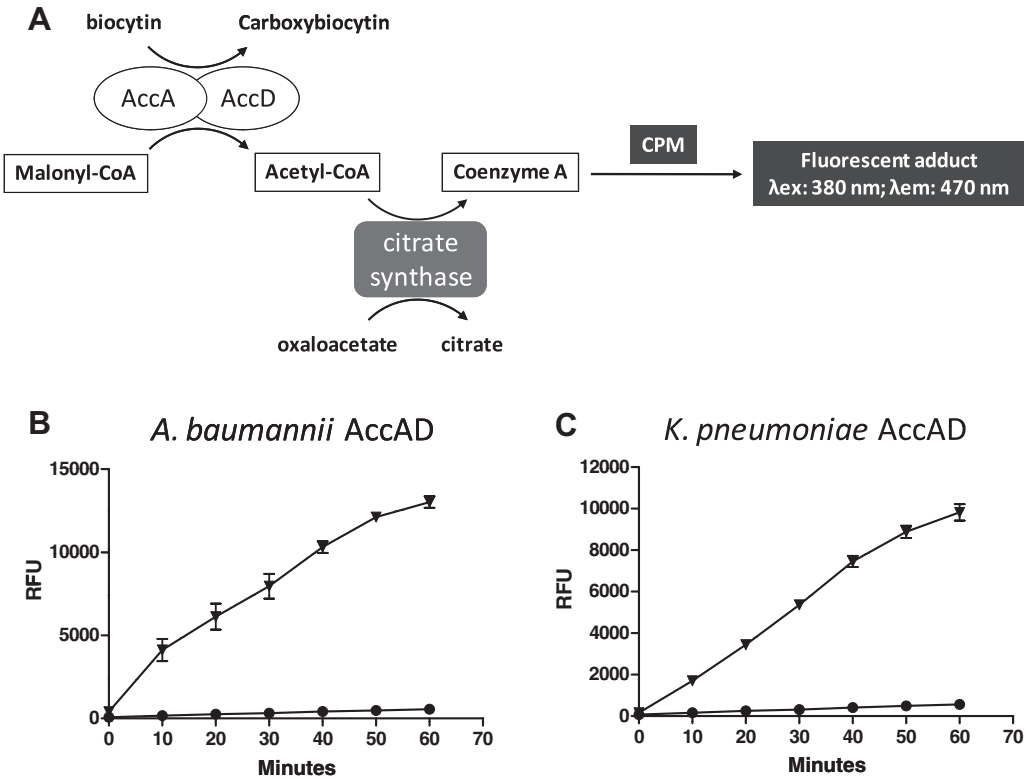

Fig.6. (A) Indirect fluorimetric assay. CoA generated by the coupling activities of AccAD and citrate synthase reacts with CPM, forming a fluorescent adduct with λ_{ex} = 384 nm and λ_{em} = 470 nm. AccAD activities from *A. baumannii* (B) and *K. pneumoniae* (C) were monitored continuously for 1 h at room temperature in the presence (▼) or absence (●) of malonyl-CoA. Results correspond to two independent experiments done in duplicate.

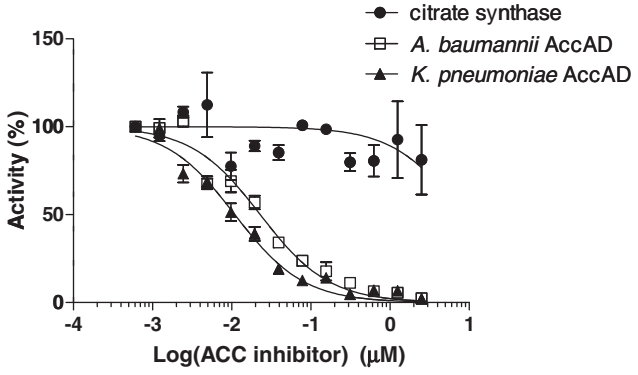

Fig.7. Inhibition dose–response curve. AccAD domains from *A. baumannii* (□) and *K. pneumoniae* (▲) were incubated with increasing concentrations of AccAD inhibitor. Citrate synthase (●) activity was not affected by increasing concentrations of AccAD inhibitor. Results are representative of two independent experiments performed in duplicate.

carbapenems that has been observed in clinical isolates from bloodstream infections. Carbapenem resistance is due to the spread of carbapenemase-producing *K. pneumoniae* (KPC) [28,39].

ACCase catalyzes the ATP-dependent carboxylation of acetyl-CoA to generate malonyl-CoA. The kinetic constants for the substrates acetyl-CoA and ATP were determined, and both *A. baumannii* and *K. pneumoniae* holoenzymes displayed similar K_M values. It is noteworthy that these values were similar to the apparent K_M constants for acetyl-CoA and ATP reported for E. coli ACCase [33]. The activity of both holoenzymes was linear for 50 min at room temperature, but the reaction time was limited to minimize substrate consumption and prevent potential malonyl-CoA-dependent inhibition [33]. Finally, the ACCases were susceptible to inhibition by nanomolar concentrations of a pseudopeptide pyrrolidinedione inhibitor [23,24].

We reported here several assay formats for monitoring holo-ACCase or isolated AccAD carboxyltransferase activity. The LC–MS/MS method that we used to monitor holo-ACCase activity has two major advantages. First, it enables the direct measurement of either substrate consumption or product formation, thereby avoiding the compound interference commonly associated with enzyme-coupled assays [38,40]. Second, the increased sensitivity of the LC–MS/MS assay allows the use of lower enzyme concentrations, thereby reducing reagent use. However, because the LC–MS/MS cycle time is several minutes per sample, this assay readout is not suitable for high-throughput compound screening.

The second holo-ACCase readout we developed here is an ATP depletion assay based on the ATP detection reagent Kinase-Glo Plus. It is noteworthy that the acetyl-CoA K_M values obtained with this ATP depletion readout were similar to those obtained with the LC–MS/MS method. This ATP depletion assay has the advantage of being easily miniaturized to 384- and 1536-well formats for higher throughput compound screening.

Finally, we reported here a modified reverse carboxyltransferase assay for monitoring the catalytic activity of the isolated AccAD subunit. Specifically, instead of using the colorimetric thiol detection reagent DTNB, we used the fluorogenic thiol detection reagent CPM. In general, assays with a fluorescent readout are better suited for compound screening because the occurrence of compound interference with the assay readout is reduced. Using this assay format, we demonstrated that purified recombinant AccAD from *A. baumannii* and *K. pneumoniae* were active as isolated subunits and susceptible to inhibition by a pseudopeptide pyrrolidinedione inhibitor.

The activity assays and enzyme characterization reported here could provide the basis for high-throughput compound screening to identify novel ACCase inhibitors. Specifically, the ATP depletion assay could be used for high-throughput compound screening; the LC–MS/MS assay could then be used to confirm apparent inhibitors, and the isolated AccAD assay could be used to determine whether hit compounds inhibit the CT (AccAD) or BC (AccC) subunit. Compounds identified by such a screening strategy might be useful as lead compounds for development of novel antibiotics for treatment of Gram-negative bacterial infections.

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