INFECTION AND IMMUNITY, Apr. 1987, p. 848–855
0019-9567/87/040848-08$02.00/0

Vol. 55, No. 4

Cloning and Functional Expression of the *Coxiella burnetii* Citrate Synthase Gene in *Escherichia coli*

ROBERT A. HEINZEN* AND LOUIS P. MALLAVIA

Department of Microbiology, Washington State University, Pullman, Washington 99164-4340

Received 2 September 1986/Accepted 4 December 1986

The citrate synthase gene from the obligate intracellular rickettsial parasite *Coxiella burnetii* was cloned and expressed in *Escherichia coli*. Transduction into *E. coli* with a *C. burnetii* gene library constructed in the cosmid vector pHK17 resulted in the functional complementation of the *gltA* mutation of *E. coli* MOB154. A GltA$^+$ clone carrying 16.4 kilobase pairs of *C. burnetii* DNA and designated pJCC959 was isolated and characterized. Southern hybridization analysis confirmed that the pJCC959 cloned insert consists of *C. burnetii* DNA and that homology exists with the *Rickettsia prowazekii* citrate synthase gene. Subcloning analysis with the multicopy expression vector pUC8 revealed that citrate synthase expression was under control of a *C. burnetii* promoter. In vitro transcription-translation of subclones pLPM20 and pLPM30 established a molecular weight of ca. 46,000 for the monomer form of the cloned enzyme. Transposon Tn*5* mutagenesis of pLPM30 defined the coding region to approximately 1.2 kilobase pairs of *C. burnetii* DNA. Maxicell analysis of selected pLPM30::Tn*5* insertion derivatives identified the direction of transcription and the relative translational start and stop sites and substantiated the molecular weight value calculated from the in vitro analysis. Inhibition studies showed that citrate synthase activity in crude cell extracts obtained from strain MOB154 transformed with the cloned *C. burnetii* gene was markedly inhibited by 4 mM ATP, while 4 mM α-ketoglutarate had virtually no effect. These data indicate that the *C. burnetii* enzyme displays regulatory behavior characteristic of the small gram-positive bacterial and eucaryotic enzyme.

Coxiella burnetii is an obligate intracellular rickettsial parasite which infects a variety of invertebrate and vertebrate hosts and causes Q fever in humans (2, 13). The organism is able to remain viable extracellularly for many years (2), but replication will occur only within the harsh, acidic environment of host cell phagolysosomes (10). The acidophilic nature of *C. burnetii* has been demonstrated by incubating organisms in axenic buffer adjusted to pH 4.5 (the approximate intraphagolysosomal pH), which stimulates transport, incorporation, and catabolism of various substrates (16–20). At pH 7, *C. burnetii* exists essentially in a state of metabolic quiescence.

By virtue of the obligate intracellular niche of this rickettsia, traditional biochemical and genetic analyses are difficult. No established metabolic mutants exist, and evidence regarding utilization of classical biochemical pathways, such as the Embden-Meyerhof pathway of glycolysis and the citric acid cycle, has been gathered by analyzing low-level oxidations of various substrates by cell lysates or intact organisms (2, 19, 27).

To help eludicate basic metabolic processes, it was desirable to clone *C. burnetii* genes into a more easily manipulated organism, such as *Escherichia coli*. The first enzyme of the citric acid cycle, citrate synthase [citrate(*si*)-synthase; EC 4.1.3.7], is the rate-limiting enzyme of the cycle (24), catalyzing the condensation reaction between acetyl coenzyme A (acetyl-CoA) and oxaloacetate (OAA) with the liberation of citrate. It was selected for cloning studies because of its key metabolic role in biosynthesis and energy production. Additionally, it has been demonstrated that in axenic buffer at pH 4.5, *C. burnetii* preferentially transports and metabolizes citric acid cycle intermediates and precursors such as pyruvate, succinate, glutamate, and proline (16–20). From this it was reasoned that the citrate synthase gene (*gltA*) is probably constitutively transcribed in *C. burnetii* from a strong promoter and is therefore a likely candidate for expression in *E. coli*.

Citrate synthases from numerous sources have been shown, with few exceptions, to fall into two primary classes (39). Gram-positive bacteria and eucaryotic cells produce a small, dimeric enzyme with a subunit size of M_r 45,000 to 60,000 (39) that is isosterically inhibited by ATP (21) and insensitive to NADH inhibition (40). Conversely, gram-negative bacteria produce a large enzyme consisting of four to six subunits of M_r 45,000 to 60,000 each (39), which is not inhibited by ATP (21) but exhibits allosteric inhibition by NADH (40). Additionally, facultative anaerobes of this group, such as *E. coli*, show allosteric inhibition by α-ketoglutarate (α-KG) (43). These criteria have been used to help define the phylogenetic status of various bacteria (39).

The citrate synthase gene has been cloned from *Rickettsia prowazekii*, and the enzyme has been purified and characterized (29, 42). While typically considered a gram-negative bacterium, *R. prowazekii* produces an enzyme with regulatory behavior characteristic of the small gram-positive bacterial and eucaryotic enzyme. The cloning of the *C. burnetii* citrate synthase gene described in this report allowed for a comparison between the genes and gene products of these two obligate intracellular parasites. In addition, it demonstrates that a *C. burnetii* gene is functionally expressed in *E. coli*.

(A preliminary account of these findings has been presented [R. A. Heinzen and L. P. Mallavia, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, D55, p. 75].)

* Corresponding author.

TABLE 1. Bacterial strains, plasmids, and phage

Strains, plasmids, and phage	Genotype[a]	Relevant phenotype[a] and use	Source or reference
E. coli			
HB101		Enzyme analysis	Maniatis et al. (26)
JEF8	*thr-31 carB8 relA1 metB1* λ− *spoT1*	Su−, λ−, for λ::Tn*5* mutagenesis	Mergeay et al. (28)
Q1	*thr-1 leu-6 thi-1 lacY1 supE44 tonA21*	SupE; to grow λ::Tn*5*	De Bruijn and Lupski (12)
MOB154	*gltA6 galK30 pyrD36 relA1 rpsL129 thi-1 supE44 hsdR4 recA* λ⁻	GltA⁻	Wood et al.[b]
MS327	*araD* Δ(*lac pro*) *argE*am *rif nal recA56*	Maxicell analysis	Sancar et al. (33)
C. burnetii			
Nine Mile RSA493	Ph I	Source of cloned genomic DNA, Southern analysis	Samuel et al. (32)
Dugway 7E9-12	Ph I	Southern analysis	Stoenner et al. (36)
Plasmids			
pHK17	Kn^r Tc^r	Cloning vector containing λ *cos* sequence	Klee et al. (23)
pUC8	Ap^r	Subcloning analysis	Vieira and Messing (37)
pUC19	Ap^r	Subcloning analysis	Vieira and Messing (37)
pBR322	Ap^r Tc^r	Probe in Southern analysis	Bolivar et al. (8)
pMW212	Ap^r	pBR322 derivative containing *R. prowazekii gltA* gene	Wood et al.[b]
pJCC959	Kn^r Tc^r	pHK17 derivative; GltA$^+$	This work
pLPM10	Ap^r	*Eco*RI subclone of pJCC959; GltA$^+$	This work
pLPM20	Ap^r	*Eco*RI-*Pst*I subclone of pLPM10; GltA$^+$	This work
pLPM30	Ap^r	*Eco*RI-*Hin*cII subclone of pLPM10; GltA$^+$	This work
pLPM40	Ap^r	pUC19 derivative of pLPM30; GltA$^+$	This work
Phage λ467	λ *b221*, *rex*::Tn*5*, *c*I857 *O*am29, *P*am80	λ::Tn*5*; for Tn*5* mutagenesis	Kleckner et al. (22)

[a] Abbreviations: Tc^r, tetracyline resistance; Kn^r, kanamycin resistance; Ap^r, ampicillin resistance; +, positive; −, negative; Ph I, phase I.
[b] Strain MOB154 is a *hsdR4 recA* variant of *E. coli* W620, (42) constructed by D. Wood. Plasmid pMW212 is a subclone of pMW150 (42).

MATERIALS AND METHODS

Chemicals and enzymes. All reagents are commercially available and were obtained from Sigma Chemical Co., St. Louis, Mo.; Bio-Rad Laboratories, Richmond, Calif.; Bethesda Research Laboratories, Gaithersburg, Md.; New England BioLabs, Beverly, Mass.; and Difco Laboratories, Detroit, Mich.

Bacterial strains, plasmids, and bacteriophage. The bacterial strains, plasmids, and phage used in this work are listed in Table 1. All *E. coli* strains are derivatives of *E. coli* K-12.

Media and growth conditions. Luria-Bertani (LB) broth was used as the complex growth medium for *E. coli* strains and has been previously described (26). For selection of GltA$^+$ clones, the minimal medium of Vogel and Bonner (38) was used with 0.5% glucose as the sole carbon source. Supplements of thiamine hydrochloride (20 μg/μl), uracil (40 μg/μl) and L-glutamate (2 mM) were added as required. Agar was added to a concentration of 1.5% for solid media. Appropriate antibiotic selection was done for plasmid DNA with tetracycline, kanamycin, and ampicillin at final concentrations of 12.5, 25, and 100 μg/ml, respectively. Kanamycin was used at a concentration of 100 μg/ml when selection for Tn*5* insertions was done.

***C. burnetii* propagation and purification.** *C. burnetii* strains used in this study were acquired from the Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Mont. Rickettsial suspensions of infected yolk sacs were inoculated into 6-day-old, embryonated white Leghorn eggs (H and N Hatchery, Redmond, Wash.). Infected yolk sacs of dying chicken embryos were harvested, and *C. burnetii* was purified as described previously (20).

Manipulation of chromosomal and plasmid DNA. Chromosomal DNA was purified from *C. burnetii* Nine Mile Ph I cells by a previously published procedure (31). Plasmid DNA was isolated from *E. coli* cells by the alkaline extraction method of Birnboim and Doly (5). Covalently closed circular DNA was purified from cell lysates by CsCl-ethidium bromide buoyant density gradient centrifugation (26).

Restriction endonucleases were used under the conditions recommended by the suppliers. Preparation of a gene library was done by partially digesting *C. burnetii* Nine Mile Ph I genomic DNA with *Eco*RI, collecting 0.5% agarose gel size-fractionated fragments (26) in the 20- to 40-kilobase-pair (kbp) range, and ligating these fragments to *Eco*RI-digested pHK17 (23). All ligation reactions were performed at 16°C for 24 h by using an excess of T4 DNA ligase and under conditions that optimize insert-to-vehicle ligation (26). Clones were isolated by screening recombinant plasmid DNA (5) in *E. coli* MOB154 (Table 1) colonies exhibiting the appropriate phenotype. Restriction endonuclease mapping and electrophoretic analysis of plasmids and restriction products were done by previously described methods (26).

Packaging of pHK17 recombinants was done in vitro with a freeze-thaw lysate prepared as described previously (26). Subsequent transductions and transformations of *E. coli* were done essentially by the calcium chloride method (11).

Probe preparation and nitrocellulose filter blot hybridization. Preparation of nick-translated DNA probes, DNA-DNA hybridizations, and autoradiography were done as previously described (31). Transfer of DNA samples to nitrocellulose filters from 0.8% agarose gels was done either by the method of Southern (35) or by electrophoresis at 1.75 mA in 25 mM phosphate buffer for 2 h (6).

Tn*5* mutagenesis. Cultures of *E. coli* JEF8 (Su$^-$) harboring pLPM30 were mutagenized with λ467 (22) at a multiplicity of

infection of 10 by the method described by De Bruijn and Lupski (12). Mutagenized plasmid DNA was isolated from these cells and used to transform GltA$^-$ *E. coli* MOB154. Insertional inactivation of the *gltA* coding region was assayed by the inability of pLPM30::Tn*5* derivatives to functionally complement strain MOB154 and allow for growth on glucose minimal media containing kanamycin and ampicillin.

In vitro and in vivo labeling of proteins. For in vitro analysis of cloned proteins, plasmid DNA was added to a bacterial DNA-directed cell-free transcription-translation system purchased from Enzo Biochem, Inc., New York, N.Y. Protein labeling was done according to instructions sent by the supplier with 30 μCi of [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) at a specific activity of 400 to 500 Ci/nmol. In vivo labeling of plasmid-encoded proteins was done essentially by the procedure of Sancar et al. (33) with the *E. coli* maxicell strain MS327 transformed with plasmid DNA. [^{35}S]methionine (150 μCi; specific activity, 1,131 Ci/nmol) was added to labeling medium and allowed to incorporate into protein. In vitro and in vivo samples were subjected to sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (25), and labeled protein was visualized by fluorography (9).

Enzyme analysis. Crude cell extracts used in citrate synthase assays were prepared, by the method described by Wood et al. (42), from stationary-phase *E. coli* cells propagated in LB medium under appropriate antibiotic selection. Extracts were prepared from *C. burnetii* cells in the same manner, with the exception of the cell fractionation step, which was done with a Braun MSK model cell homogenizer (B. Braun Instruments, Burlingame, Calif.). Specifically, 44 mg (dry weight) of *C. burnetii* Nine Mile Ph I cells suspended in 15 ml of 40 mM potassium phosphate buffer (pH 7.4) was added to a fractionation bottle containing 20 g of acid-washed no. 13 Ballotini glass beads (0.17 to 0.18 mm) which had previously been cooled to 0°C. This concentrated rickettsial suspension was homogenized for 4.5 min under carbon dioxide cooling. The glass beads were then pelleted by spinning the fractionation bottle at 500 rpm for 2 min in a Beckman JA-14 rotor; the supernatant, containing broken and unbroken cells, was decanted; and the unbroken cells were pelleted as described by Wood et al. (42). Typical protein concentrations as measured by the Bio-Rad assay were 3 to 6 μg/μl for *E. coli* and 1 to 2 μg/μl for *C. burnetii*. The protein standard consisted of purified, recrystallized bovine serum albumin.

Citrate synthase was assayed spectrophotometrically by the method of Sere (34) with a Beckman 25 spectrophotometer. Increase in A_{412} was monitored by using 5,5′-dithiobis(2-nitrobenzoate) to measure the appearance of free sulfhydryl groups of released coenzyme A moieties. The standard reaction mixture contained 0.05 mM acetyl-CoA, 0.5 mM OAA, 118 mM Tris hydrocholoride (pH 8.1), 0.1 mM 5,5′-dithiobis(2-nitrobenzoate), and 50 to 200 μg of *E. coli* cell extract or 400 to 600 μg of *C. burnetii* cell extract in a final volume of 1.5 ml. All assays were done at room temperature (21°C). A preincubation period preceded initiation of the reaction with OAA to account for any OAA-independent activity as described previously (34). When desired, addition of the effectors α-KG or ATP at 4 mM was done before reaction initiation. Inhibition by NADH was not investigated owing to the presence in crude cell extract preparations of malate dehydrogenase, which catalyzes the production of OAA while consuming NADH. Enzyme specific activities are expressed as milli-international units per milligram of protein and were derived from initial reaction velocities.

RESULTS

Isolation of *gltA* on pHK17. The 12.9-kbp plasmid pHK17 (23) is a broad-host-range vector containing the genes for kanamycin (Km^r) and tetracycline (Tc^r) resistance and also the lambda *cos* sequence, which allows for in vitro packaging of this vector, containing 20 to 40 kbp of insert DNA, into lambda heads. A *C. burnetii* Nine Mile Ph I gene library constructed in pHK17 was used to directly transduce the citrate synthase (*gltA*) mutant *E. coli* MOB154. This strain is unable to grow on glucose minimal medium unless provided with a nutritional supplement of glutamate (15). Functionally expressing GltA$^+$ clones were selected by scoring for growth on glucose minimal medium agar plates containing kanamycin and tetracycline. Plating of an estimated 170 pHK17-*C. burnetii* recombinants yielded three positive clones. Restriction endonuclease analysis of plasmid DNA revealed that each pHK17 recombinant had in common two *Eco*RI insert fragments of 7.9 and 6.2 kbp in size (data not shown). Because the clone designated pJCC959 contained the smallest insert, 16.4 kbp, it was chosen for partial restriction mapping (Fig. 1) and further subcloning analysis.

Subcloning of *gltA* and homology with *R. prowazekii*. Insert DNA was subcloned into a multicopy plasmid expression vector to facilitate plasmid DNA manipulations and to further define the *gltA* complementing region within the cloned 16.4-kbp *C. burnetii* fragment of pJCC959. The pUC family of plasmids was chosen for their high copy number and polylinker regions, which contain numerous unique restriction sites (37). The subclones which were subsequently generated, pLPM10 (10.6 kbp), pLPM20 (7.9 kbp), pLPM30 (6.0 kbp), and pLPM40 (6.0 kbp), were all GltA$^+$ as assayed by their ability to complement strain MOB154, thereby allowing for growth on glucose minimal media containing ampicillin. All functional subclones were derived from the same 7.9-kbp *Eco*RI cloned fragment of pJCC959 (Fig. 1).

Citrate synthase gene activity was seen irrespective of the relative orientation of insert DNA and was evidenced by pLPM20 and pLPM30 insert orientation versus pLPM10 and pLPM40 insert orientation (Fig. 1). pLPM40 has the identical insert of pLPM30, except in the opposite orientation with respect to the pUC β-galactosidase (*lacZ*) promoter and was constructed for comparison of enzyme activity.

Southern analysis confirmed that the transforming quality of the clones resides in *C. burnetii* genomic DNA. Restriction endonuclease-digested chromosomal and plasmid DNAs were subjected to agarose gel electrophoresis (Fig. 2a), transferred to nitrocellulose, and probed with nick-translated pLPM30. As demonstrated by autoradiography, a ^{32}P-labeled pLPM30 probe hybridizes to 7.9-kbp *Eco*RI fragments in both *C. burnetii* Nine Mile Ph I and Dugway strain chromosomal DNA (Fig. 2b, lanes A and B) and also to the corresponding 7.9-kbp cloned *Eco*RI fragment in pJCC959 (Fig. 2b, lane C). The expected homologies with pLPM10, pLPM20, and the controls pLPM30 and pUC8 (Fig. 2b, lanes D, E, F, and G) are also seen with pUC8 vector homology, represented by a signal migrating at approximately 2.7 kbp. No homology was observed with any cloned DNA or *C. burnetii* genomic DNA when pUC8 was used as a probe (data not shown).

To determine whether homology exists between the previously cloned *R. prowazekii* citrate synthase gene and the

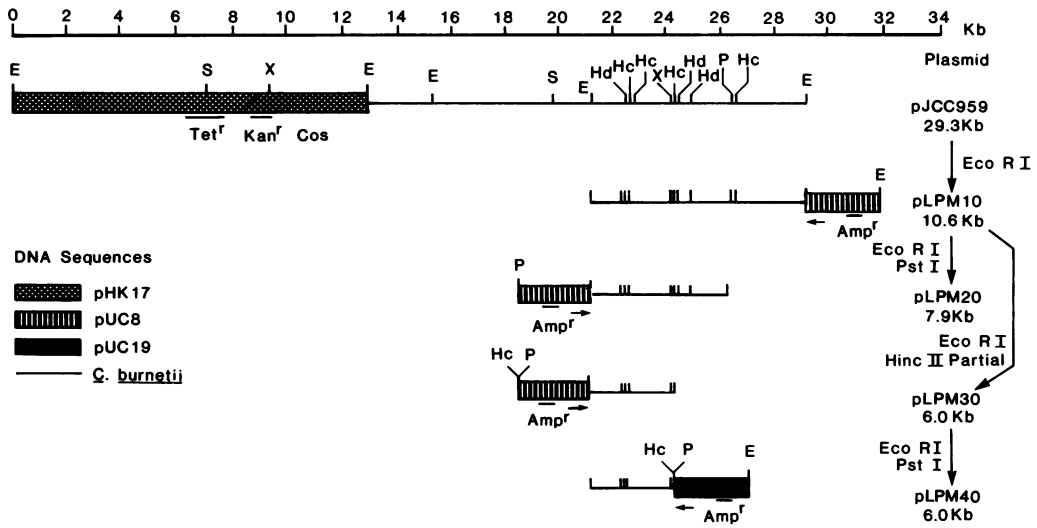

FIG. 1. Subcloning analysis of the *C. burnetii gltA* region. All clones are phenotypically GltA$^+$. Only some restriction sites are shown in vector DNA, with the 7.9-kbp cloned *Eco*RI fragment of pJCC959 mapped with the enzymes *Hin*dIII and *Hin*cII. *C. burnetii* DNA restriction sites in pUC subclones are vertically aligned with the corresponding sites present in *C. burnetii* DNA in pJCC959. The *lacZ* promoter of pUC8 and pUC19 is represented by an arrow below each of the maps. Abbreviations: E, *Eco*RI; Hc, *Hin*cII; Hd, *Hin*dIII; P, *Pst*I; S, *Sal*1; X, *Xho*I; Amp^r, ampicillin resistance; Kan^r, kanamycin resistance; Tet^r, tetracycline resistance; Cos, cohered phage λ cohesive ends.

C. burnetii gltA gene, a Southern analysis was performed with nick-translated pMW212 (a pBR322 construction with a 2.7-kbp insert containing the *R. prowazekii gltA* gene) (Table 1) to probe *Eco*RI-digested pJCC959 (Fig. 3a, lane B). Initial exposure identified homology between the Tc^r regions contained in the vector portions of the two clones (Fig. 3b, lane B). Extended exposure reveals an additional weak signal from the pJCC959 7.9-kbp *Eco*RI fragment used in the generation of all GltA$^+$ subclones (Fig. 3c, lane B). This latter homology is not seen when pBR322 is used as a probe (data not shown).

Identification of plasmid-encoded proteins. To visualize proteins encoded by GltA$^+$ pUC8 clones, an in vitro *E. coli* cell-extract-directed transcription-translation system was used followed by fluorography. Figure 4, lane B, shows proteins coded for by a colE1 plasmid control. The proteins produced by pUC8 include a 28,000-molecular-weight β-lactamase encoded by the *bla* gene (14) that was seen as a major band (Fig. 4, lane C). pLPM20 and pLPM30, in addition to the *bla* gene product, exhibit a major unique protein migrating at approximately molecular weight 46,000

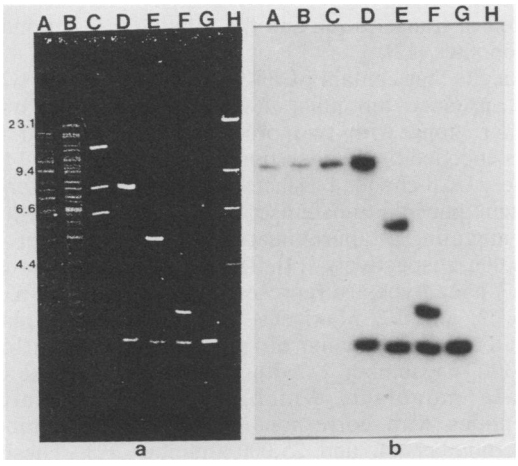

FIG. 2. Homology of pLPM30 with *C. burnetii* chromosome. (a) Agarose gel (0.8%) showing endonuclease digests of *C. burnetii* Nine Mile Ph I chromosomal DNA (*Eco*RI) (lane A), *C. burnetii* Dugway chromosomal DNA (*Eco*RI) (lane B), pJCC959 (*Eco*RI) (lane C), pLPM10 (*Eco*RI), (lane D), pLPM20 (*Eco*RI-*Pst*I) (lane E), pLPM30 (*Eco*RI-*Pst*I (lane F), pUC8 (*Eco*RI (lane G), phage λ (*Hin*dIII) (lane H). (b) Autoradiograph of DNA in panel (a) after electrophoretic transfer to nitrocellulose and hybridization with ^{32}P-labeled pLPM30 with 0.3× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) at 68°C that allows for approximately a 7% mismatch.

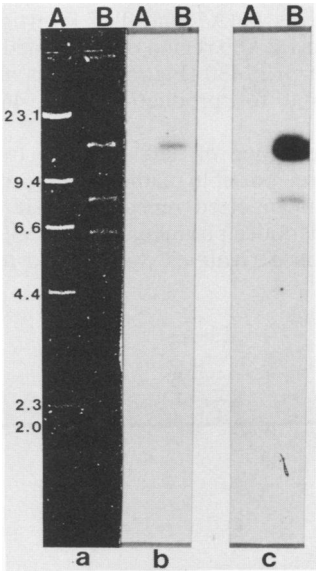

FIG. 3. Homology of pJCC959 with *R. prowazekii* citrate synthase gene. (a) Agarose gel (0.8%) showing endonuclease digests of phage λ (*Hin*dIII) (lane A) pJCC959 (*Eco*RI) (lane B). (b) Autoradiograph of DNA in panel (a) after transfer to nitrocellulose by the method of Southern (35) and hybridization with ^{32}P-labeled pMW212 with 6× SSC at 68°C that allows for approximately a 32% mismatch. Exposure was for 2 h. (c) As for panel (b). Exposure was for 17 h.

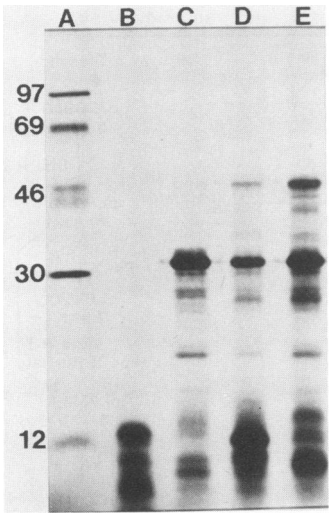

FIG. 4. In vitro transcription-translation of plasmid-encoded proteins. Proteins were labeled with [^{35}S]methionine, separated on a sodium dodecyl sulfate-12.5% polyacrylamide gel, and fluorographed. Lanes: A, ^{14}C-labeled standards expressed in kilodaltons; B, ColE1 plasmid control; C, pUC8; D, pLPM20; E, pLPM30.

(Fig. 4, lanes D and E). This protein, unique to GltA$^+$ clones, has a molecular weight which correlates with that of other established citrate synthase monomers (39), suggesting that this band represents the *C. burnetii gltA* gene product. Bands of weak intensity are presumably partially translated polypeptides.

Characterization of *gltA* coding region. To more specifically define the region encoding the 46,000-dalton *C. burnetii* citrate synthase monomer, pLPM30 was mutagenized with transposon Tn*5*. Transpositions into a structural gene will abolish gene function through insertional inactivation. All mutants scored as GltA$^-$ had Tn*5* insertions localized to approximately a 1.2-kbp region of the cloned 3.3-kbp *Eco*RI-*Hinc*II insert of pLPM30 (Fig. 5). This is about the coding capacity required for production of a 46,000-molecular-weight protein.

The DNA sequence of Tn*5* contains translational stop codons in all three possible reading frames within the first 30 base pairs of its inverted repeats (1). Hence, analysis of truncated polypeptides produced in maxicells harboring Tn*5* plasmid insertion derivatives can be used to determine the

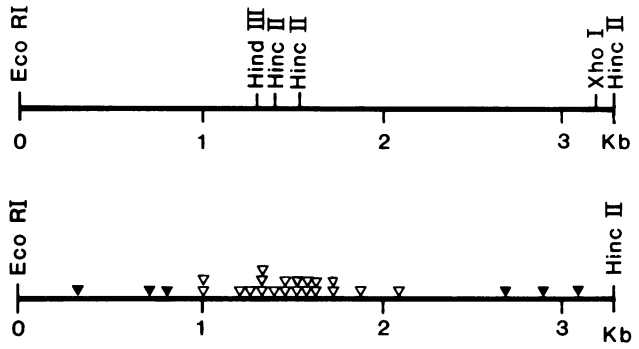

FIG. 5. Restriction site and Tn*5* insertion map of the *C. burnetii gltA* region contained in the 3.3-kbp cloned insert DNA of pLPM30. Symbols: ▼, GltA$^+$ Tn*5* insertion; ▽, GltA$^-$ Tn*5* insertion.

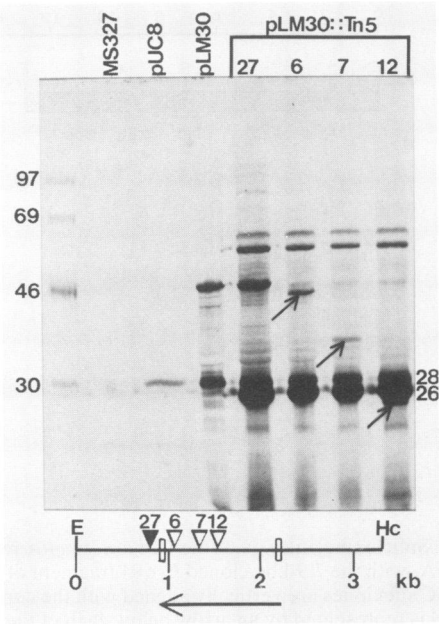

FIG. 6. Maxicell analysis of truncated polypeptides produced by pLPM30::Tn*5* derivatives. Polypeptides synthesized by UV-irradiated MS327 maxicells containing pLPM30::Tn*5* derivatives were labeled with [^{35}S]methionine, separated on a sodium dodecyl sulfate-12.5% polyacrylamide gel, and fluorographed. ^{14}C-labeled standards expressed in kilodaltons are shown in the left-hand lane; the other lanes contain polypeptides as marked. The β-lactamase (28 kilodaltons) and neomycin phosphotransferase II (26 kilodaltons) are indicated as 28 and 26, respectively. Truncated citrate synthase monomers are indicated by diagonal arrows. The map at the bottom of the figure shows the position of Tn*5* insertions in the 3.3-kbp insert of pLPM30 and the direction of *gltA* transcription. The relative translational start and stop sites are shown as open boxes. Insertion number 27 confers a GltA$^+$ phenotype, and insertion numbers 6, 7, and 12 confer a GltA$^-$ phenotype.

direction of transcription and the relative translational start and stop sites (12).

Maxicells that contain pLPM30::Tn*5* number 27, which is GltA$^+$, produce an intact 46,000-dalton citrate synthase monomer along with two proteins of 54,000 and 49,000 daltons encoded by the insertion sequences of Tn*5* (14) (Fig. 6). The pUC8-encoded β-lactamase and the Tn*5*-encoded neomycin phosphotransferase type II are represented by bands migrating at approximately molecular weight 28,000 and 26,000, respectively (14). Tn*5* insertions which result in a GltA$^-$ phenotype are represented by pLPM30::Tn*5* numbers 6, 7, and 12. Maxicells that harbor these plasmids directed the synthesis of polypeptides identical to pLPM30::Tn*5* number 27 with the exception of the citrate synthase monomer, which has been truncated to polypeptides with corresponding M_r 44,500 (number 6), 39,200 (number 7), and 25,000 (number 12). These data establish the direction of transcription as shown by the arrow at the bottom of Fig. 6. Also, assuming an average molecular weight of 125 for an amino acid, the relative translational start and stop sites were calculated and are shown as open boxes (Fig. 6).

Enzyme analysis. Citrate synthase activity observed in crude cell extracts prepared from *C. burnetii* Nine Mile Ph I cells and from the GltA$^-$ *E. coli* MOB154 complemented with the cloned *C. burnetii* citrate synthase gene displayed regulatory properties opposite to those seen with extracts

TABLE 2. Inhibitory effects of ATP and α-KG on citrate synthase activity

Strain	Citrate synthase[a]		
	Sp act (mIU/mg)	Effect of following inhibitor (% sp act of control)[a]:	
		4mM ATP	4mM α-KG
E. coli K-12			
HB101	3.7	129.0	33.6
MOB154	0.0	ND[b]	ND
MOB154 (pJCC959)	13.5	23.6	102.8
MOB154 (pLPM10)	26.0	18.2	96.5
MOB154 (pLPM20)	193.6	15.9	108.3
MOB154 (pLPM30)	213.8	9.2	103.9
MOB154 (pLPM40)	206.9	10.2	106.4
C. burnetii Nine Mile Ph I	1.7	52.7	101.5

[a] Mean value of two or three determinations.
[b] ND, Not determined.

derived from the GltA$^+$ *E. coli* HB101 (Table 2). The HB101 enzyme was markedly inhibited by 4 mM α-KG. Only 33.6% of the control activity was seen in the presence of this modulator. Rickettsial enzyme activity was not affected by 4 mM α-KG. In contrast, the *C. burnetii* enzyme was greatly inhibited by 4 mM ATP, while the HB101 enzyme was slightly stimulated. The discrepancy observed between the level of ATP inhibition of the cloned and uncloned *C. burnetii* enzyme (9.2 to 23.6% versus 52.7% of the activity of the control) may be due to the inhibitor being effectively titrated out by ATP-binding proteins or ATPase activity present in the large amount of total protein (400 to 600 mg) used in assays performed with extracts of Nine Mile Ph I cells. However, if the concentration of ATP is raised to 16 mM in assays run on extracts prepared from rickettsial cells, only 26% of the control activity is observed (data not shown).

It is interesting that as the size of successive subclones decreased, the specific activity increased, as exemplified by pLPM10 (10.6 kbp; specific activity, 13.5) compared with pLPM30 (6.0 kbp; specific activity, 213.8). The basis for the increased GltA activity was not determined but could be related to the deletion of a negative regulatory element or could simply be the result of an increase in plasmid copy number.

Using Lineweaver-Burk double-reciprocal plots, we calculated the apparent acetyl-CoA K_m values for the cloned enzyme (pJCC959) and the enzyme prepared from *C. burnetii* Nine Mile Ph I cells. Values established were 24.6 and 20.7 μM, respectively, and saturation was hyperbolic in nature.

DISCUSSION

This report describes the first cloning and functional expression of a *C. burnetii* gene in *E. coli*. The *C. burnetii* citrate synthase gene was isolated by complementing an established *E. coli* GltA$^-$ mutant with a *C. burnetii* cosmid gene library. In vivo and in vitro protein labeling identified a *C. burnetii* DNA sequence which encodes a 46,000-molecular-weight protein that represents the citrate synthase monomer form. Subcloning analysis and Tn*5* mutagenesis localized the coding region to a 1.2-kbp interval contained in the 3.3-kbp *Eco*RI-*Hin*cII *C. burnetii* DNA insert in pLPM30.

Evidence that *E. coli* is utilizing a rickettsial promoter was established by subcloning insert DNA into the pUC polylinker region in both orientations with respect to the *lacZ* promoter. GltA activity was seen in either orientation. Additionally, the specific activities from pLPM30 (213.8 mIU/mg) and pLPM40 (206.9 mIU/mg) were not significantly different. Further proof of *C. burnetii* transcriptional promotion was shown by maxicell analysis of truncated polypeptides produced by pLPM30::Tn*5* derivatives. The direction of *gltA* transcription was determined to be opposite that of the pUC8-encoded *lacZ* gene (37), indicating that GltA activity was not the result of a transcriptional or translational fusion.

C. burnetii is morphologically similar to gram-negative bacteria in that it has an outer membrane containing lipopolysaccharide (2). Surprisingly, however, it produces a citrate synthase with regulatory properties typifying those of the small enzyme associated with gram-positive bacteria and eucaryotic cells. A physiological level of ATP (4 mM) strongly inhibited *C. burnetii* citrate synthase activity, while no sensitivity to inhibition by 4 mM α-KG was observed. Additionally, the *C. burnetii* enzyme demonstrates hyperbolic saturation for acetyl-CoA with an apparent K_m of approximately 22 μM. These data are in agreement with those established for gram-positive bacterial and eucaryotic enzymes and contrast markedly with those seen for typical free-living gram-negative bacteria such as *E. coli*, for which the acetyl-CoA K_m value is much higher (ca. 400 μm) and saturation is sigmoidal in nature (39). Of interest, *R. prowazekii*, another gram-negative intracellular parasite, produces a citrate synthase that also displays characteristics similar to those of the small type enzyme (29, 42).

Southern analysis demonstrated limited homology between the *gltA* genes of *R. prowazekii* and *C. burnetii*. This may indicate active site or subunit interaction site homologies with a considerable degree of nonhomology in regions not critical to functionality.

The amino acid sequence of the small, dimeric porcine heart citrate synthase is known (7), and residues involved in catalysis and intersubunit interaction have been identified (30). A comparison with the amino acid sequence of the large, multimeric *E. coli* enzyme reveals that, although there are considerable regions which demonstrate no homology, there are, distributed throughout the sequence, apparently significant homologies with some conservation of residues important in substrate binding and catalysis (3, 4). Also, Wood, using the deduced amino acid sequence from a cloned fragment of the *R. prowazekii* citrate synthase gene, has identified homologies with both the *E. coli* and the porcine heart enzyme active sites (D. O. Wood, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, D51, p. 74). This lends credence to the speculation that evolutionarily, the two types of enzyme have maintained the same basic active-site structure necessary to perform the reaction while diverging to accommodate different effectors. Studies are currently under way to sequence the *C. burnetii* citrate synthase gene to allow for a more detailed comparison with the *R. prowazekii gltA* gene.

The reason two gram-negative obligate intracellular parasites have evolved to produce a gram-positive-type enzyme sensitive to ATP inhibition appears to be related to the characteristic physiology of the cell regarding energy production. As suggested by Phibbs and Winkler, the adenylate modulation of citrate synthase and hence the control of the energy-producing function of the citric acid cycle by *R. prowazekii* may be advantageous to the organism, consider-

ing its ability to transport ATP from the host cytoplasm in exchange for rickettsial ADP (29, 41). The preference shown by *C. burnetii* for substrates directly metabolized by the citric acid cycle may demonstrate an important, if not essential, energy-producing role of this pathway. Modulation of citrate synthase by ATP, which can be considered the ultimate end product of the oxidative mode of the citric acid cycle, may therefore be the most effective method of directly controlling the energy needs of the cell.

Cloning of *C. burnetii* genes into *E. coli* can circumvent many problems associated with research on this rickettsia. Isolation of cell constituents is arduous and time-consuming. This report demonstrates a greater than 100-fold increase in *C. burnetii* citrate synthase specific activity when the gene is carried on a multicopy plasmid (Table 2). This methodology may therefore be applied as an initial purification step in projects designed to analyze proteins key to the virulence and peculiar intracellular location and behavior of *C. burnetii*.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI20190 from the National Institutes of Health.

We thank D. Wood for graciously supplying *E. coli* MOB154 and pMW212. We are grateful to Jim Samuel and Laura Hendrix for critical review of this manuscript and to Cheryl Stone and Cynthia Hulse for help in its preparation.

LITERATURE CITED

1. **Auerswald, E. A., G. Ludwig, and H. Schaller.** 1981. Structural analysis of Tn5. Cold Spring Harbor Symp. Quant. Biol. **45**:107–113.
2. **Baca, O. G., and D. Paretsky.** 1983. Q-fever and *Coxiella burnetii*: a model for host-parasite interactions. Microbiol. Rev. **47**:127–149.
3. **Bell, W. A., V. Bhayana, and H. W. Duckworth.** 1983. Evidence for structural homology between the subunits from allosteric and nonallosteric citrate synthase. Biochemistry **22**:3400–3405.
4. **Bhayama, V., and H. Duckworth.** 1984. Amino acid sequence of *Escherichia coli* citrate synthase. Biochemistry **23**:2900–2905.
5. **Birnboim, H. D., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
6. **Bittner, M., P. Kupferer, and C. F. Morris.** 1980. Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyloxymethyl cellulose or nitrocellulose sheets. Anal. Biochem. **102**:459–471.
7. **Bloxham, D. P., D. C. Parmelee, S. Kumar, K. A. Walsh, and K. Titani.** 1982. Complete amino acid sequence of porcine heart citrate synthase. Biochemistry **21**:2028–2036.
8. **Bolivar, F., R. L. Rodriguez, P. J. Greere, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
9. **Bonner, W., and A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids. Eur. J. Biochem. **46**:83–88.
10. **Burton, P. R., J. Stuckemann, R. M. Welsh, and D. Paretsky.** 1978. Some ultrastructural effects on persistent infections by the rickettsia *Coxiella burnetii* in mouse L-cells and green monkey kidney (VERO) cells. Infect. Immun. **21**:556–566.
11. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **De Bruijn, F. J., and J. Lupski.** 1984. The use of transposon Tn5 in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids—a review. Gene **27**:131–149.
13. **Derrick, E. H.** 1937. "Q" fever, a new fever entity: clinical features, diagnosis and laboratory investigation. Med. J. Aust. **2**:281–299.
14. **Gassler, M. G., R. H. A. Plasterk, and P. van de Putte.** 1982. G inversions in bacteriophage Mu: a novel way of gene splicing. Nature (London) **297**:339–342.
15. **Gilvarg, C., and B. Davis.** 1956. The role of the tricarboxylic acid cycle in acetate oxidation in *Escherichia coli*. J. Biol. Chem. **222**:307–319.
16. **Hackstadt, T., and J. C. Williams.** 1981. Biochemical strategem for obligate parasitism of eukaryotic cells by *Coxiella burnetii*. Proc. Natl. Acad. Sci. USA **78**:3240–3244.
17. **Hackstadt, T., and J. C. Williams.** 1981. Incorporation of macromolecular precursors by *Coxiella burnetii* in an axenic medium, p. 431–440. *In* W. Burgdorfer and R. L. Anacker (ed.), Rickettsiae and rickettsial diseases. Academic Press, Inc., New York.
18. **Hackstadt, T., and J. C. Williams.** 1981. Stability of the adenosine 5′-triphosphate pool on *Coxiella burnetii*: influence of pH and substrate. J. Bacteriol. **148**:419–425.
19. **Hackstadt, T., and J. C. Williams.** 1983. pH dependence of the *Coxiella burnetii* glutamate transport system. J. Bacteriol. **154**:598–603.
20. **Hendrix, L., and L. P. Mallavia.** 1983. Active transport of proline by *Coxiella burnetii*. J. Gen. Microbiol. **130**:2857–2863.
21. **Jangaard, N. O., J. Unkeless, and D. E. Atkinson.** 1968. The inhibition of citrate synthase by ATP. Biochim. Biophys. Acta **151**:225–235.
22. **Kleckner, N., R. K. Chan, B. Tye, and D. Bostein.** 1975. Mutagenesis by insertion of a drug-resistance element carrying an inverted repetition. J. Mol. Biol. **97**:561–575.
23. **Klee, H. J., G. P. Milton, and E. W. Nester.** 1983. Complementation analysis of *Agrobacterium tumefaciens* Ti plasmid mutations affecting oncogenicity. J. Bacteriol. **150**:327–331.
24. **Krebs, H. A., and J. M. Lowenstein.** 1960. The tricarboxylic acid cycle, p. 129–203. *In* D. M. Greenberg (ed.), Metabolic pathways. Academic Press, Inc., New York.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **McDonald, T. L., and L. Mallavia.** 1971. Biochemistry of *Coxiella burnetii*: Embden-Meyerhof pathway. J. Bacteriol. **107**:864–869.
28. **Mergeay, M., D. Gigot, J. Beckman, N. Glansdorf, and A. Pierard.** 1974. Physiology and genetics of carbamoyl phosphate synthesis in *Escherichia coli* K12. Mol. Gen. Genet. **133**:299–316.
29. **Phibbs, P. V., Jr., and H. H. Winkler.** 1982. Regulatory properties of citrate synthase from *Rickettsia prowazekii*. J. Bacteriol. **149**:718–725.
30. **Remington, S., G. Wiegand, and R. Huber.** 1982. Crystallographic refinement and atomic models of two different forms of citrate synthase at 2.7 and 1.7 Å resolution. J. Mol. Biol. **158**:111–152.
31. **Samuel, J. E., M. E. Frazier, M. L. Kahn, L. S. Thomashow, and L. P. Mallavia.** 1983. Isolation and characterization of a plasmid from phase I *Coxiella burnetii*. Infect. Immun. **41**:488–493.
32. **Samuel, J. E., M. E. Frazier, and L. P. Mallavia.** 1985. Correlation of plasmid type and disease caused by *Coxiella burnetii*. Infect. Immun. **49**:775–779.
33. **Sancar, A., R. P. Wharton, S. Seltzer, B. Kacinski, N. D. Clarke, and W. D. Rupp.** 1981. Identification of *uvrA* gene product. J. Mol. Biol. **148**:45–62.
34. **Sere, P. A.** 1969. Citrate synthase. Methods Enzymol. **13**:3–11.
35. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
36. **Stoenner, H. G., and D. B. Lackman.** 1960. The biologic properties of *Coxiella burnetii* isolated from rodents collected in Utah. Am. J. Hyg. **71**:45–51.
37. **Vieira, J., and J. Messing.** 1983. The pUC plasmids, an M13

mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
38. **Vogel, J. H., and D. M. Bonner.** 1956. Acetylornithase of *Escherichia coli*. Partial purification and some properties. J. Biol. Chem. **218**:97–106.
39. **Weitzman, P. D. J., and M. J. Danson.** 1976. Citrate synthase. Curr. Top. Cell. Regul. **10**:161–204.
40. **Weitzman, P. D. J., and D. Jones.** 1968. Regulation of citrate synthase and microbial taxonomy. Nature (London) **219**:270–272.
41. **Winkler, H. H.** 1976. Rickettsial permeability: an ADP-ATP transport system. J. Biol. Chem. **22**:389–396.
42. **Wood, D. O., W. H. Atkinson, R. S. Sikorski, and H. Winkler.** 1983. Expression of the *Rickettsia prowazekii* citrate synthase gene in *Escherichia coli*. J. Bacteriol. **155**:412–416.
43. **Wright, J. A., P. Maeba, and B. D. Sanwal.** 1967. Allosteric regulation of the activity of citrate synthase of *Escherichia coli* by α-ketoglutarate. Biochem. Biophys. Res. Commun. **29**: 34–38.