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Expression of the *Rickettsia prowazekii* Citrate Synthase Gene in *Escherichia coli*

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Recombinant DNA techniques were used to isolate the *Rickettsia prowazekii* citrate synthase gene on the plasmid vector pBR322 by functional complementation of a *gltA* mutation of *Escherichia coli* K-12. Analysis of citrate synthase activity in crude extracts revealed that the enzyme expressed in *E. coli* retains the regulatory control mechanisms characteristic of the rickettsial enzyme.

Rickettsia prowazekii, the etiological agent of epidemic typhus, is an obligate, intracellular parasitic bacterium. This organism, like other members of the genus *Rickettsia*, occupies a unique bacterial niche in that it grows directly within the cytoplasm of the eucaryotic host cell, unbounded by a vacuolar membrane (27). Genetic and biochemical analyses of rickettsial metabolism, host cell interactions, and pathogenicity are hampered by the difficulties associated with growing sufficient rickettsiae in eggs and cell cultures for many biochemical studies and by a lack of rickettsial mutants and identified gene transfer mechanisms. One approach for circumventing these difficulties would be to introduce *R. prowazekii* genes into *Escherichia coli*, where the genes and gene products could be examined in a more manageable system.

To examine rickettsial gene expression in *E. coli*, we chose to clone the gene for an intracellular enzyme that appears to have an important regulatory role in *R. prowazekii* metabolism. Citrate synthase (EC 4.1.3.7) is the first enzyme of the citric acid cycle. It catalyzes the formation of citrate from acetyl coenzyme A and oxaloacetate. This enzyme has been studied extensively in a variety of procaryotic and eucaryotic cells (28), and recently the rickettsial enzyme was partially purified by Phibbs and Winkler (19). Citrate synthase from *R. prowazekii* is similar in a number of properties to the enzymes isolated from gram-positive bacteria and eucaryotic cells. In addition, it is strongly inhibited by ATP. In contrast, *E. coli* citrate synthase is not inhibited by ATP (12), but is strongly inhibited by α-ketoglutarate (αKG) (30). *E. coli* mutants deficient in citrate synthase (*gltA*) have been isolated (1, 9), and the functional cloned gene has been identified in the Clarke-Carbon colony bank (10).

This paper describes the isolation, by functional complementation of a *gltA* mutation in *E. coli*, of the *R. prowazekii* citrate synthase gene. A preliminary report of this work has been presented and appears in abstract form in *Plasmid* (in press).

The bacterial strains used are listed in Table 1. The Madrid E strain of *R. prowazekii* was propagated in antibiotic-free, embryonated hen eggs, harvested from the yolk sacs, and purified as described previously (29). The complex medium used for growth of *E. coli* strains was LB (8), containing 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract (Difco), and 5 g of NaCl per liter at a final pH of 7.0. The minimal medium (VB) was that described by Vogel and Bonner (26) and contained 0.5% (wt/vol) glucose or arabinose and the following supplements as needed: uracil, 40 μg/ml; thiamine hydrochloride, 2 μg/ml; and L-glutamic acid, 2 mM. For solid media, agar was added to 1.5% (wt/vol). For selection, ampicillin, tetracycline, and streptomycin were added to final concentrations of 50, 25, and 100 μg/ml, respectively.

Cesium chloride (technical grade) was obtained from Kawecki Berylco Industries, New York, N.Y. Dithiobis-(2-nitrobenzoate) was purchased from Calbiochem, La Jolla, Calif. Lysozyme (grade I), protease (type V), RNase A, ATP, ethidium bromide, oxaloacetic acid, acetyl coenzyme A, ampicillin, tetracycline, and streptomycin were obtained from Sigma Chemical Co., St. Louis, Mo. Agarose, dithiothreitol, restriction endonucleases, T4 DNA ligase, and bacterial alkaline phosphatase were obtained from Bethesda Research Laboratories, Rockville, Md.

DNA was isolated from the Madrid E strain of *R. prowazekii* by the Marmur procedure (17).

TABLE 1. Bacterial strains[a]

Strain	Description	Source/reference
R. prowazekii Madrid E	Avirulent	5
E. coli	F⁻ *hsdS20 ara-14*	
RR1(pBR322)	*proA2 leuB6 lacY1 galK2 rpsL20 thi-1 xyl-5 mtl-1 supE44* λ⁻ carrying pBR322	J. Gaubatz[b] (3)
C-1	F⁻, modification negative	CGSC[c] 3121
HB94	Hfr *hsdR4 thi-1 relA1 gal-55* λ⁻ *spoT1*	CGSC 5461 (4)
SK472	Hfr *relA1 spoT1 thi-1 zjj-202*::Tn*10 serB22* λ⁻	CGSC 6102
AB1623	F⁻ *gltA5 lct-1 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 tsx-57 tfr-5 rpsL20 supE44*	CGSC 1623 (1)
W620,gal$_7$⁻	F⁻ *gltA6 galK30 pyrD36 relA1 rpsL129 thi-1* λ⁻ *supE44*	CGSC 4278 (21)
MOB145	HB94 derivative containing *zjj-202*::Tn*10*	This study
MOB147	*hsdR* derivative of W620,gal$_7$⁻	This study
MOB150	*hsdR* Ara⁺ derivative of AB1623	This study
MOB155	MOB150 carrying pMW150	This study

[a] Genotype symbols are described by Bachmann and Low (2) and Davis et al. (8). pBR322 confers resistance to ampicillin and tetracycline.

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[c] *E. coli* Genetic Stock Center, B. Bachmann, Department of Human Genetics, Yale University School of Medicine, New Haven, Conn.

The final solvent for all DNAs was TE buffer (10 mM Tris-hydrochloride, 1 mM EDTA [pH 8.0]). Plasmid DNA was isolated from 300 ml of *E. coli* cultures grown in LB broth containing ampicillin. Amplification with chloramphenicol was performed as described by Maniatis et al. (16). Lysis of the *E. coli* cells and preparation of a cleared lysate was accomplished by a modification of the method of Clewell and Helinski (6). Covalently closed circular plasmid DNA was purified from cleared lysates by cesium chloride-ethidium bromide equilibrium density gradient centrifugation (20). Ethidium bromide was removed from the plasmids by extraction with butanol saturated with 5 M NaCl, 1 mM EDTA, and 10 mM Tris-hydrochloride (pH 8.0), and the DNA was dialyzed against TE buffer.

One microgram of pBR322 DNA in TM buffer (90 mM Tris-hydrochloride [pH 7.4], 10 mM $MgCl_2$) was digested to completion with restriction endonuclease *Bam*HI. The reaction was terminated by heat inactivation for 10 min at 65°C. Bacterial alkaline phosphatase (78 U) was added, and the incubation was continued for 15 min at 37°C. This reaction was terminated by the addition of 30 μl of phenol saturated with TM buffer. The mixture was centrifuged, and the aqueous layer was collected and extracted three times with ether saturated with TM buffer. The phosphatase-treated pBR322 DNA was then mixed with 4 μg of *R. prowazekii* DNA partially digested with *Sau*3A. This DNA mixture was placed on ice, and the following were added: 50 mM MgCl (10 μl), 0.1 M dithiothreitol (10 μl), 0.5 mM ATP (10 μl), and water (20 μl). To this final volume (100 μl), 1 U of T4 DNA ligase was added, and the mixture was incubated at 4°C for 18 h.

To prevent the destruction of entering *R. prowazekii* DNA, an *hsdR* mutation, which eliminates *E. coli* K-12 host restriction activity, was introduced into the *E. coli gltA* recipient strains by classical methods of *E. coli* conjugation and transduction (7). MOB150 was constructed by conjugally transferring *hsdR* to AB1623 from HB94, an HFr strain that transfers the chromosome beginning near the *hsd* locus. Selection was made for arabinose utilization and streptomycin resistance. Transconjugants were screened for acquisition of *hsdR* by their sensitivity to λ *vir* bacteriophage (kindly provided by David Friedman) propagated on *E. coli* C-1. Due to a lack of selectable markers, it was impossible to conjugally transfer *hsdR* from HB94 to W620. Therefore, transposon Tn*10* was transferred from SK472 by P1 *vir* (kindly provided by June Scott) transduction and positioned next to the *hsdR* gene of HB94 by legitimate recombinational events. Transfer of the *hsdR4* gene could then be detected by following conjugal transfer of tetracycline resistance to W620. Selection was made on LB agar medium containing tetracycline and streptomycin. Transconjugants were screened for coinheritance of *hsd* by the λ *vir* screening test described above. To allow the use of vector-coded tetracycline resistance during cloning experiments, a tetracycline-sensitive variant (MOB147) was isolated by the techniques of Maloy and Nunn (15).

E. coli mutants deficient in citrate synthase

are unable to grow on glucose minimal medium unless supplemented with glutamate (9, 10). Therefore, to select for cells acquiring a functional gene, $CaCl_2$-treated cells of MOB150 (*gltA hsdR*), transformed with pBR322-*R. prowazekii* DNA ligation mixtures by the method described by Davis et al. (8), were transferred to glucose minimal medium containing ampicillin. From a total of 10 independent ligations and transformations, 12 transformants were observed after a 3-day incubation at 37°C. Control samples plated on LB agar with ampicillin revealed a frequency of transformation for these experiments of approximately 2×10^3 ampicillin-resistant transformants per μg of pBR322 DNA.

Since the *gltA* mutation of MOB150 is not a deletion, it was necessary to differentiate between a clone containing a functional *R. prowazekii* gene and a Glt^+ revertant of MOB150. Plasmid DNA was isolated from nine of the transformants by a rapid, small-volume technique (11) and was used to transform separate samples of competent MOB150 cells. Selection was for ampicillin-resistant transformants. Each ampicillin-resistant colony was then transferred to glucose minimal medium to check for coinheritance of the *gltA* gene. For one of the isolates, all ampicillin-resistant transformants also grew on glucose minimal medium.

This clone, designated MOB155, was selected for further study. MOB155 had a generation time of approximately 4.5 h when grown in VB plus glucose and ampicillin. The generation time was decreased to 1.6 h with the addition of glutamic acid. Purified plasmid DNA from MOB155, when transformed into two different *gltA* strains (MOB147 and MOB150), always demonstrated coinheritance of ampicillin resistance and the ability to grow on glucose minimal medium.

By comparison of migrations with known standards (14) in agarose gels, the plasmid isolated from MOB155, designated pMW150, was found to have a molecular mass of approximately 6.4×10^6 daltons. Since pBR322 has a molecular mass of 2.9×10^6 daltons (25), approximately 3.5×10^6 daltons of rickettsial DNA is inserted into the *Bam*HI site of pBR322. Restriction endonuclease digestions of pMW150 revealed that pMW150 is cleaved once by *Eco*RI, *Sal*I, and *Ava*I at sites within the vector portion of the molecule, whereas there are at least two *Hin*dIII sites within the inserted rickettsial DNA.

To demonstrate that the inserted DNA came from *R. prowazekii*, ^{32}P-labeled pMW150 was used to probe *R. prowazekii* and *E. coli* DNAs (Fig. 1). pMW150 failed to hybridize detectably with *E. coli* chromosomal DNA (Fig. 1B, lane 2). Increased sensitivity was obtained with the small *Eco*RI-*Hin*dIII digestion fragment of plasmid pLC26-17. This plasmid was obtained from the Clarke-Carbon colony bank and consists of a ColE1 vector and a fragment of *E. coli* chromosomal DNA carrying the *E. coli* citrate synthase gene (10). Digestion of pLC26-17 with *Eco*RI and *Hin*dIII releases a small fragment which almost entirely consists of the *E. coli* citrate synthase gene. Although the labeled probe hybridized with the vector fragment due to the homology between ColE1 and pBR322, it failed to hybridize with the small fragment containing the *E. coli* citrate synthase gene (Fig. 1B, lane 3). The probe did hybridize with an *Eco*RI fragment of *R. prowazekii* chromosomal DNA (Fig. 1B, lane 1). This demonstrates that the inserted DNA is rickettsial in origin.

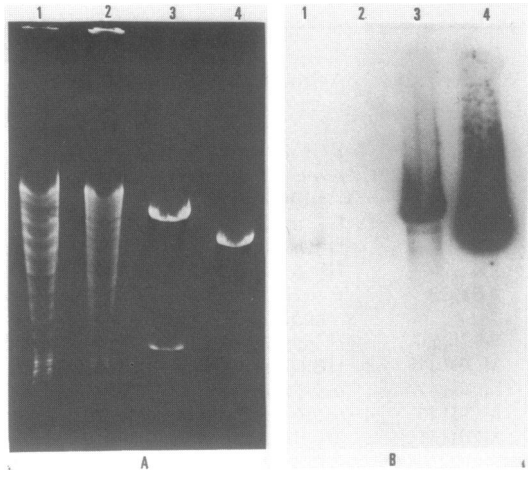

FIG. 1. Hybridization of pMW150 plasmid DNA with *R. prowazekii* and *E. coli* chromosomal DNA and with plasmid pLC26-17. Unlabeled chromosomal and plasmid DNAs were digested with restriction endonucleases, electrophoresed through 0.8% agarose, denatured, and transferred to nitrocellulose paper. Probe plasmid DNA was pMW150 ^{32}P labeled by nick translation (22), using a nick translation kit supplied by New England Nuclear Corp., Boston, Mass. Transfer of DNA from gels to nitrocellulose filter paper and hybridization with a labeled probe were performed by the method of Southern (23). (A) Ethidium bromide-stained agarose gel before transfer. Lane 1, *Eco*RI-digested *R. prowazekii* chromosomal DNA (Madrid E); lane 2, *Eco*RI-digested *E. coli* chromosomal DNA (HB94); lane 3, *Eco*RI-plus-*Hin*dIII-digested pLC26-17 (the smaller fragment contains the citrate synthase gene of *E. coli*); lane 4; *Eco*RI-digested pMW150. (B) Autoradiograph of *R. prowazekii*, *E. coli*, pLC26-17, and pMW150 DNAs from the gel in (A) after hybridization with ^{32}P-labeled pMW150 DNA.

Additional proof that the *E. coli* Glt^+ clone is expressing a rickettsial citrate synthase gene came from an examination of the regulatory properties of the cloned enzyme. Cell extracts

from *R. prowazekii* (Madrid E), *E. coli* HB94 (Glt^+), and *E. coli* MOB155 (containing the *R. prowazekii* gene for citrate synthase) were assayed for citrate synthase activity and for sensitivity to the effectors αKG and ATP. The enzyme from *E. coli* was extremely sensitive to inhibition by 4 mM αKG (Table 2). Both the rickettsial enzyme and the enzyme derived from MOB155 were insensitive to such inhibition. The rickettsial enzyme was inhibited by 4 mM ATP, as was the cloned enzyme (Table 2). However, the *E. coli* enzyme was slightly stimulated by ATP under these conditions. These data clearly indicate that the cloned citrate synthase has regulatory properties, with respect to αKG and ATP, that are similar to the rickettsial enzyme and unlike that of *E. coli*.

The results reported here demonstrate for the first time that an *R. prowazekii* gene can be isolated and expressed in an *E. coli* K-12 host. In this case, the *R. prowazekii* citrate synthase gene was identified by its ability to functionally complement a mutant of *E. coli* deficient in the enzyme.

TABLE 2. Effect of αKG and ATP on citrate synthase activity[a]

Cell extract source	Sp act (mIU/mg)	Sp act with effector (% of control)	
		Plus αKG (4 mM)	Plus ATP (4 mM)
R. prowazekii	17 ± 3 (8)[b]	98 ± 8 (9)	43 ± 18 (9)
Clone MOB155	11 ± 2 (7)	110 ± 12 (8)	60 ± 4 (10)
E. coli HB94	24 ± 9 (7)	11 ± 7 (10)	117 ± 34 (17)

[a] Cells were collected by centrifugation, washed twice, and suspended in 40 mM potassium phosphate buffer (pH 7.4). Cell suspensions were disrupted by passage through a French pressure cell at 20,000 lb/in^2. The resulting lysate was centrifuged at 12,000 × *g* for 10 min to pellet unbroken cells and clarified by centrifugation at 105,000 × *g* for 120 min. This supernatant was then extensively dialyzed against a solution of 2.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4, 1 mM EDTA, and 10 mM magnesium chloride (pH 7.5). The resulting cell extracts contained from 2 to 12 mg of protein per ml. Citrate synthase (citrate oxaloacetate-lyase [coenzyme A-acetylating], EC 4.1.3.7) activity was assayed spectrophotometrically by the method of Srere (24). The reaction mixture contained 0.1 mM 5,5′-dithiobis-(2-nitrobenzoate), 0.05 mM acetyl coenzyme A, 0.5 mM oxaloacetate, 118 mM Tris-hydrochloride (pH 8.1), and 50 to 140 μg of cell-free extract in a final volume of 1.0 ml. Reactions were initiated with oxaloacetate, and this rate was corrected for any activity observed in the absence of oxaloacetate. The effectors (αKG) and ATP, when included, were added before and after the addition of oxaloacetate, respectively, at a concentration of 4 mM. Specific activities (milli-international units per milligram) were calculated, and inhibition data were expressed as percentages of control activity. Protein was determined by the method of Lowry et al. (13).

[b] Mean and standard deviation; number of determinations in parentheses.

Citrate synthase from *R. prowazekii* has been partially purified by Phibbs and Winkler, and its molecular weight and regulatory properties have been described previously (18, 19). Although rickettsiae are morphologically typical of gram-negative bacteria, their citrate synthase had a molecular weight of 62,000, which is atypical for gram-negative bacteria (28). The rickettsial enzyme was also insensitive to NADH and αKG, but was sensitive to inhibition by adenylates (ATP > ADP > AMP). The inhibition by adenylates was competitive with respect to acetyl coenzyme A. The citrate synthase from *R. prowazekii* was thus not typical of the enzyme from other gram-negative bacteria, but was more like the "small" type found in gram-positive bacteria and eucaryotic cells (19). We obtained similar results with the cloned *R. prowazekii* citrate synthase. Analysis of crude extracts revealed that the enzyme coded for by the cloned gene retained the regulatory control mechanisms exhibited by the rickettsial enzyme for ATP and αKG. NADH sensitivities were not assayed because the crude extracts contained malate dehydrogenase, which, in the presence of NADH, consumed oxaloacetic acid from the reaction mixture. The retention by the cloned enzyme of its rickettsial regulatory mechanisms within *E. coli* might contribute to the slower growth rate of the clone. The growth rate, however, is more than sufficient for the rickettsial citrate synthase to be isolated easily and in large quantities for biochemical characterization.

The methods employed in this paper could be used to clone any rickettsial gene for which a corresponding mutant of *E. coli* exists, provided the rickettsial gene is expressed. In addition, clone banks for the entire genome can be established and screened for rickettsial activities. In this way, *R. prowazekii* genes and gene products related to the intracellular existence, antigenicity, and pathogenicity of this organism are made available for genetic and biochemical analyses.

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