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Characterization of Activity and Expression of Isocitrate Lyase in *Mycobacterium avium* and *Mycobacterium tuberculosis*

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Analysis by two-dimensional gel electrophoresis revealed that *Mycobacterium avium* expresses several proteins unique to an intracellular infection. One abundant protein with an apparent molecular mass of 50 kDa was isolated, and the N-terminal sequence was determined. It matches a sequence in the *M. tuberculosis* database (Sanger) with similarity to the enzyme isocitrate lyase of both *Corynebacterium glutamicum* and *Rhodococcus fascians*. Only marginal similarity was observed between this open reading frame (ORF) (termed *icl*) and a second distinct ORF (named *aceA*) which exhibits a low similarity to other isocitrate lyases. Both ORFs can be found as distinct genes in the various mycobacterial databases recently published. Isocitrate lyase is a key enzyme in the glyoxylate cycle and is essential as an anapleurotic enzyme for growth on acetate and certain fatty acids as carbon source. In this study we express and purify Icl, as well as AceA proteins, and show that both exhibit isocitrate lyase activity. Various known inhibitors for isocitrate lyase were effective. Furthermore, we present evidence that in both *M. avium* and *M. tuberculosis* the production and activity of the isocitrate lyase is enhanced under minimal growth conditions when supplemented with acetate or palmitate.

Most microorganisms growing on acetate or fatty acids as the sole carbon source employ the glyoxylate bypass for the biosynthesis of cellular material. The key enzymes of this bypass are isocitrate lyase and malate synthase (18). The former cleaves isocitrate to succinate and glyoxylate, and the latter condenses glyoxylate with acetyl coenzyme A (acetyl-CoA) to yield malate. The glyoxylate bypass circumvents the loss of two carbon dioxides of the tricarboxylic acid cycle (TCA cycle), thereby permitting net incorporation of carbon into cellular structures during growth on acetate. In addition, even during operation of the TCA cycle, many fatty acids are partially metabolized to acetyl-CoA, thus requiring the presence of isocitrate lyase (for a review, see reference 37).

Isocitrate lyase competes with the TCA cycle enzyme isocitrate dehydrogenase for their common substrate isocitrate. By changing the total cellular activity of either of the two enzymes and/or by changing their affinities toward isocitrate, control of carbon flux between the two cycles is achieved (22). In *Escherichia coli* growth on acetate leads to a decrease in $NADP^+$-dependent isocitrate dehydrogenase activity caused by the reversible phosphorylation of isocitrate dehydrogenase. The corresponding isocitrate dehydrogenase-kinase is encoded in the same operon as the isocitrate lyase and the malate synthase. The reduction in isocitrate dehydrogenase activity redirects isocitrate into the glyoxylate cycle through the activity of isocitrate lyase. The phosphorylation-dephosphorylation of isocitrate dehydrogenase is believed to regulate entry of the substrate into the glyoxylate bypass (26, 39). In addition, *E. coli* isocitrate lyase is inhibited by several metabolites, e.g., succinate, 3-phosphoglycerate, or phosphoenolpyruvate, leading to a more subtle control of the carbon flux (23, 30).

In mycobacteria, isocitrate lyase activity has been reported to increase continuously with the age of the culture in *Mycobacterium tuberculosis* $H_{37}R_v$ (25) but not in *M. tuberculosis* $H_{37}R_a$ or *M. smegmatis* (34). Other studies report enhanced glyoxylate cycle enzyme activity under low oxygen tension (41) or when the mycobacteria are grown in the presence of acetate (15).

Previous studies in our laboratory employing two-dimensional (2-D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE analysis of *M. avium* have identified a 50-kDa polypeptide, the expression of which was markedly upregulated upon infection of macrophages (35). N-terminal sequencing of the protein showed 15 amino acid residues, 13 of which were identical to the *aceA* (Rv 0467) sequence in *M. tuberculosis* databases. Upon examination of the databases, we found another open reading frame (ORF) that is identified as an isocitrate lyase, namely, *ace*A (4). In $H_{37}R_v$, this ORF has a single-base-pair overlap resulting in two ORFs (*aceAa*/Rv1915 and *aceAb*/Rv1916; Sanger database), while it is read as one continuous ORF in CSU93 (MT1966; The Institute for Genomic Research database). For ease of reference and in light of the current findings, we refer to Rv0467 as *icl* (for isocitrate lyase) to distinguish it from the latter ORF, which we refer to as *aceA*.

To investigate whether both genes gave rise to active enzymes, we cloned the ORFs based on the DNA sequence of CSU93 and overexpressed the proteins in *Escherichia coli*. The proteins were purified by the 6His-tag attached to the N terminus. Enzyme assays with the purified proteins indicated that both function as isocitrate lyases.

We also demonstrate here at both the protein and enzyme levels that the expression of isocitrate lyase in *M. avium* and *M. tuberculosis* increases when acetate or palmitate are the limiting carbon sources and that cultivation in the presence of succinate suppresses isocitrate lyase expression. Preliminary experiments with a polyclonal antibody raised against recombinant AceA indicate that it is expressed under similar conditions as Icl only in *M. tuberculosis* CSU93 and not in *M. tuberculosis* $H_{37}R_v$. In addition, the differences in K_m and V_{max}

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between the two enzymes suggest that AceA fulfills a role subordinate to Icl in the processing of isocitrate.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Sigma Chemical Company, St. Louis, Mo., and were of the highest purity. MTS was purchased from Promega, Madison, Wis., and the lactate dehydrogenase was obtained from Calbiochem-Novabiochem Corp., La Jolla, Calif.

Organisms and growth conditions. Experiments were conducted with *M. avium* 101, *M. tuberculosis* CSU93, and *M. tuberculosis* $H_{37}R_v$. The media used included Middlebrook 7H9 medium (Difco) containing 10% (vol/vol) OADC enrichment medium and 0.05% Tween 80. In addition, a modified Dubos medium was employed as a defined minimal medium, containing (per liter) 2 g of asparagine, 1 g of KH_2PO_4, 2.5 g of Na_2HPO_4, 10 mg of $MgSO_4 \cdot 7H_2O$, 50 mg of ferric ammonium citrate, 0.5 mg of $CaCl_2$, 0.1 mg of $ZnSO_4$, 0.1 mg of $CuSO_4$, and 0.05% Tween 80. For *M. tuberculosis* cultures, 0.5 g of Casitone (Difco) was added. The pH of the medium was 6.6. When required, carbon sources were added to a final concentration of 10 mM, with the exception of acetate, which was added to a maximal final concentration of 3 mM to *M. avium* cultures (see also reference 8). Nonaerated cultures were grown as a 30-ml culture in a 25-cm^2 flask without stirring. Aeration of cultures was achieved with a 50-mm Teflon-coated magnetic stirring bar in a 500-ml Erlenmeyer flask containing 100 ml of medium at a stir rate of 80 rpm.

E. coli HB101 harboring the two plasmids (see below) was grown in Luria-Bertani medium (32) with ampicillin (50 μg/ml) and kanamycin (50 μg/ml).

Preparation of cell extracts. Mycobacterial cultures were grown in the appropriate medium. Cells were harvested, washed three times with PBST (phosphate buffered saline plus 0.05% Tween 80), and resuspended in MOPS buffer (50 mM MOPS [morpholinepropane sulfonate], pH 6.8; 5 mM $MgCl_2$, 5 mM L-cysteine, 1 mM EDTA) supplemented with protease inhibitors (tosyl-L-lysine chloromethyl ketone, 100 μg/ml; pepstatine A, 50 μg/ml; leupeptine, 50 μg/ml; *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane, 50 μg/ml). The cells were disrupted with a Bead-Beater (Biospec, Bartlesville, Okla.). The supernatant was harvested after centrifugation for 30 min at 300,000 × *g*. This cell extract was frozen in liquid nitrogen and stored at −80°C.

Cloning and expression of the Icl protein. A 1.3-kb DNA fragment containing the putative *icl* gene was amplified by PCR by using the following oligonucleotide primers: 5′-AGC GCA TAT GTC TGT CGT CGG-3′ and 5′-GTC GGA TCC AGA CTA GTG GAA CTG G-3′ and *M. tuberculosis* CSU93 DNA as template. PCR amplification conditions were as recommended by the manufacturer for fragments smaller than 5 kb with a Perkin-Elmer 480 thermocycler with Advantage GC cDNA polymerase (Clontech). The amplified DNA was digested with *Nde*I and *Bam*HI and inserted into p6HisF-11d (kindly provided by Cheng-Ming Chiang, The Rockefeller University, New York, N.Y. [3]). For overexpression, *E. coli* HB101(pGP1-2) cells carrying the recombinant p6HisF-11d(*icl*) plasmid were grown to exponential phase and treated according to the method of Tabor and Richardson (36). Briefly, cells containing the plasmids were grown exponentially at 30°C in Luria-Bertani medium (32) in the presence of the required antibiotics. Overexpression of Icl was induced by shifting the temperature to 42°C for 20 min. After induction, the temperature was shifted down to 37°C for an additional 90 min, cells were harvested by centrifugation at 4°C, and cell pellets were frozen in liquid nitrogen.

Cloning of the AceA protein. The *aceA* gene was amplified by using primers 5′-TAG CAC ATA TGG CCA TCG CCG AAA CG-3′ and 5′-GAC TGG ATC CTA CGC CTC CTT CGT GAT-3′, with CSU93 DNA as the template. PCR amplification, cloning, and expression conditions were essentially as described for the Icl protein.

Purification of Icl and AceA. Cell lysates were prepared by resuspending the frozen cell pellets of a 500-ml culture [HB101(pGP1-2)] with the respective plasmid carrying *icl* or *aceA* in 30 ml of buffer 1 {50 mM NaH_2PO_4, 20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 100 mM NaCl, 10 mM imidazole; pH 8} with protease inhibitors (tosyl-L-lysine chloromethyl ketone, 100 μg/ml; pepstatine A, 50 μg/ml; leupeptine, 50 μg/ml; *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane, 50 μg/ml) and by sonicating the suspension on ice for 5 min in intervals with 30-s breaks (Biosonik III; Bronwil Scientific, Rochester, N.Y.). Cell debris and membranes were removed by centrifugation at 300,000 × *g* for 30 min. This cell extract was applied to 3 ml of a metal affinity resin (Talon; Clontech, Palo Alto, Calif.) equilibrated with buffer 1. After sample application, the column was washed with 30 ml of buffer 1, followed by a wash with 30 ml of buffer 2 (same as buffer 1 but with a pH of 5.6 and no imidazole). The His-tagged proteins were eluted from the resin with buffer 3 (same as buffer 1 plus 5% glycerol, 1 mM β-mercaptoethanol, and 300 mM imidazole). Fractions containing isocitrate lyase activity were pooled and frozen in liquid nitrogen.

Assay of isocitrate lyase. To determine the cleavage reaction of the isocitrate lyase, the glyoxylate formed during catalysis was reduced to glycolate by lactate dehydrogenase (LDH) with the concomitant oxidation of NADH (40). By using 96-well plates, 50 μl (65 μl if purified protein was examined) of MOPS buffer containing 2 U of LDH was mixed with 20 μl of cell extract (5 μl of purified protein). Then, 0.2 mM NADH was added, and the mixture was preincubated for 5 min at 37°C. After the addition of 2 mM threo D-(s) isocitrate, the mixture was incubated for 20 min at 37°C. The reaction was stopped with 4 M urea, and the decrease of the NADH was determined by adding the formazan dye MTS (MTS/PMS ratio of 100:1) at a final concentration of 0.2 mg/ml and reading the plate at 490 nm. Activities lower than 5 nmol/min/mg were considered to be below the sensitivity of the assay.

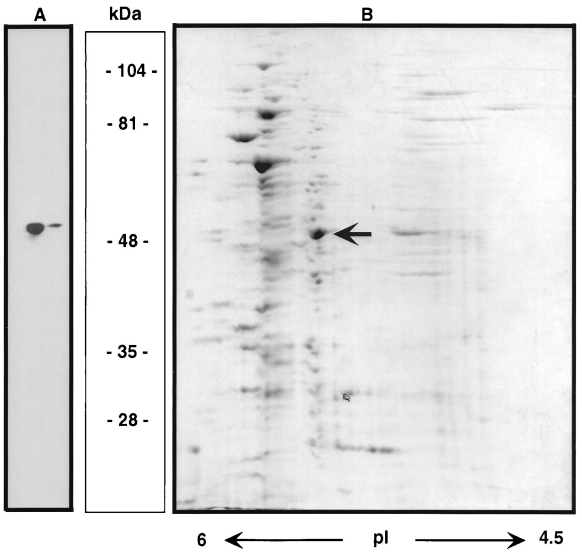


FIG. 1. 2-D PAGE of cell extracts from acetate-grown *M. avium* cultures. A total of 30 μg of crude protein extracts was applied to the gel. The Icl is indicated by an arrow. (A) Immunoblot, probed with anti-Icl antibody. (B) Coomassie blue staining. The figure also indicates the specificity of the antibody.

Determination of Michaelis and inhibition constants. The K_m and V_{max} values were determined by using a double-reciprocal Lineweaver-Burke plot. K_i values were determined from linear replots of Lineweaver-Burke slopes versus inhibitor concentrations (33).

Determination of pH optimum. Isocitrate lyase assays were performed as described above, but different buffers were used in place of 50 mM MOPS (pH 6.8). These included morpholineethanesulfonic acid-NaOH at pHs 5.5, 6, and 6.5; MOPS-NaOH at pHs 6.5, 7, and 7.5; Tricine-HCl at pHs 7.5, 8, and 8.5; and Tris-HCl at pHs 8.5 and 9.

Metal ion activity studies. The purified proteins were dialyzed against Mg^{2+}-free buffer. Isocitrate lyase assays were performed either with different metal ions or as a combination with $MgCl_2$ at a concentration of 5 mM. The metals used were $MnCl_2$, $CoCl_2$, $FeCl_2$, $CaCl_2$, $BaCl_2$, $NiCl_2$, $ZnCl_2$, $CuCl_2$, and $HgCl_2$.

Protein determination. Protein content was determined by the dye-binding method of Bradford (2).

Electrophoresis. SDS-PAGE was performed in 9% gels at 150 V by the method of Laemmli (19) by using a LKB 2050 Midget Electrophoresis Unit. 2-D electrophoresis was performed as described previously (35).

Production of polyclonal antibodies to Icl and AceA. Antibodies against the N-terminal peptide of Icl and an internal fragment of AceA (ESDDKLANV IFQPIQDRR) were produced in New Zealand White rabbits after coupling of the peptide to ovalbumin. Antibodies against AceA were also produced by injecting the purified, recombinant protein into New Zealand White rabbits. The polyclonal antibodies were affinity purified by using the respective antigen coupled to CNBr-activated Sepharose 4B.

RESULTS

N-terminal amino acid sequence. As reported previously (35), 2-D SDS-PAGE analysis of established (8- to 12-day) *M. avium* infections in murine bone marrow-derived macrophages revealed several polypeptides that were upregulated in expression when compared to bacteria grown in Middlebrook medium. The most notable of these was a protein with an apparent molecular mass of 50 kDa and an isoelectric point of approximately 5.0. This protein was transferred onto polyvinylidene difluoride membranes, and the N-terminal sequence was determined. The first 15 amino acids were found to be the following: MSVVGTPKSAEIQKD. A polyclonal antibody raised against a synthetic peptide corresponding to this amino acid sequence reacted specifically with a protein of 50 kDa in

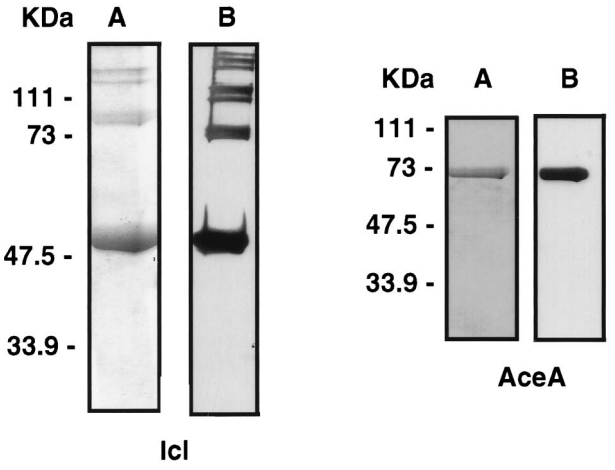


FIG. 2. SDS-PAGE of recombinant Icl and AceA. Approximately 2 μg of purified protein were applied to each lane. (A) Coomassie blue-stained SDS-PAGE gels. (B) Immunoblots with anti-Icl or anti-AceA peptide antibodies, diluted 1:5,000, respectively. The additional protein bands that also react with the antibody constitute multimers of Icl.

immunoblots of *M. avium* grown on acetate (Fig. 1). The same antibody recognized a single band of comparable mobility in SDS-PAGE-separated extracts from *M. tuberculosis* grown in macrophages and *M. leprae* isolated from SCID mice (*M. leprae* was kindly provided by J. Krahenbuhl, Louisiana State University, Carville) (data not shown).

Comparison of the amino acid sequence of the isocitrate lyase from *M. tuberculosis* with those from other organisms. The N-terminal sequence obtained from the 50-kDa protein matched a sequence of 428 amino acids in the mycobacterial genome databases (assembled by the Sanger Center, as well as by TIGR) encoded by a gene which we termed *icl* for isocitrate lyase. Further general databank searches with this ORF revealed closest similarity to the isocitrate lyases from *Corynebacterium glutamicum* and *Rhodococcus fascians* with identities to the *M. tuberculosis* sequence of 79 and 80%, respectively.

These searches also revealed a second ORF in *M. tuberculosis* with similarity to isocitrate lyase, although its level of identity with enzymes from *E. coli* (35%) and *M. tuberculosis* Icl (37%) is lower. In *M. tuberculosis* $H_{37}R_v$ this second isocitrate lyase gene (named *aceA*) consists of two overlapping ORFs (*aceAa* and *aceAb*) that share a single base pair at the 3′ end of *aceAa* and the 5′ end of *aceAb*. However, in *M. tuberculosis* CSU93 *aceA* appears to be a single ORF.

The recent progress of the sequencing projects from *M. avium*, *M. tuberculosis*, and *M. leprae* show that both the *icl* and the *aceA* ORFs exist as two distinct genes present in single copies in all of those strains, with a sequence identity of ca. 80% between species.

Expression and purification of recombinant Icl and AceA from *M. tuberculosis* CSU93 in *E. coli*. To confirm that the Icl and AceA are active isocitrate lyases, we expressed the two ORFs of *M. tuberculosis* (CSU93) in *E. coli* by using a pET-based vector. The ORFs were amplified by PCR, ligated to p6HisF-11d vector DNA, and cloned into *E. coli* HB101. The recombinant proteins were purified to approximately 90% in a one-step procedure by using a metal-affinity resin. Figure 2 shows a Coomassie blue-stained gel of the purified proteins and the corresponding Western blots. Recombinant Icl reacted with the antibody generated against the N-terminal amino acid sequence characterized in Fig. 1. The high-molecular-weight bands appear to be multimers formed in *E. coli*. We did not observe these bands on analysis of extracts from *Mycobacterium* spp. AceA reacted with an antipeptide antibody generated against an internal sequence of the *aceA* ORF. These blots confirm the identity of the recombinant enzymes and their corresponding ORFs.

Characteristics of the purified Icl. The specific activity of the purified enzyme for isocitrate was 1.3 μmol/min/mg of protein. The K_m of the purified recombinant Icl for threo D-(s) isocitrate was determined to be 145 μM by using a Hanes-Woolf plot.

The inhibition of Icl activity by several compounds, known to be effective against various isocitrate lyases, was examined. Itaconate, itaconic anhydride, bromopyruvate, and 3-nitropropionate were found to be the most potent inhibitors, with inhibition constants of 120, 190, 120, and 3 μM, respectively. Interestingly, at saturating substrate conditions succinate did not reveal any inhibitory effect on the recombinant enzyme. Oxalate and malate were also shown to inhibit the activity to approximately 50% at 5 mM inhibitor concentration. 3-Phosphoglycerate, 6-phosphogluconate, fructose-1,6-bisphosphate, and malonic acid had no inhibitory effect. Most of the effectors can be classified as structural analogs of the reaction products succinate (itaconate, itaconic anhydride, and 3-nitropropionate) or glyoxylate (3-bromopyruvate and oxalate).

In the absence of divalent cations, only negligible activity was measured for the purified Icl, whereas addition of Mg^{2+} or Mn^{2+} supported enzyme activity. Mg^{2+} at 5 mM was found to be the most effective cation. Mn^{2+} was able to replace Mg^{2+}, yielding 39% of the activity obtained with Mg^{2+}. Co^{2+}, Fe^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, and Hg^{2+} were not able to support significant Icl activity. A variety of metal ion combinations were studied for their ability to inhibit Icl activity. The cations were present in the assay mixture in an equimolar concentration with magnesium. Ca^{2+} and Ba^{2+} had no inhibitory effect. Mn^{2+} and Zn^{2+} both reduced the activity to almost 25%, while all other ions inhibited activity below the sensitivity of the assay. It is known for the isocitrate lyases of *C. glutamicum* and *Acinetobacter calcoaceticus* that Mn^{2+} can partially substitute for Mg^{2+} (12, 30).

The Icl activity was assayed for its pH dependence. Four different buffers were used in the pH range of 5.5 to 9. These buffers were used through overlapping pH values to control for possible effects of the different buffer components on Icl activity. The optimal pH for the assay of Icl activity was found to be 6.8 with a MOPS buffer.

Enzyme activity was stable for several months when the purified protein was frozen in liquid nitrogen and stored at −80°C. Even upon thawing and storage at 4°C, only minimal activity was lost after 2 months. Addition of glycerol and the presence of a reducing agent in the purification buffer appeared to be necessary to retain activity of the purified recombinant protein.

Characteristics of the purified AceA. Recombinant AceA also showed isocitrate lyase activity after purification, the K_m of the recombinant protein for threo D-(s) isocitrate was determined to be 1.3 mM by using a Tricine-HCl buffer at pH 7.5 (which was established as the optimal assay buffer for this protein). This K_m is approximately 10-fold higher than that of Icl. The V_{max} of the purified enzyme for threo D-(s) isocitrate was 0.41 μmol/min/mg, ca. three times slower than Icl. The inhibitor profile of AceA was similar to that of the Icl protein. Itaconate, itaconic anhydride, bromopyruvate, and 3-nitropropionate were effective with K_i values of 220, 480, 710, and 110 μM, respectively. Succinate inhibited the activity to approximately 50% at a 5 mM inhibitor concentration. The purified enzyme proved to be very unstable, losing its activity within a

TABLE 1. Isocitrate lyase activity in *M. avium* grown on various carbon substrates[a]

Carbon source	Mean enzyme sp act (nmol/min/mg of protein) ± SD
Palmitate	1,193.4 ± 0.2
Acetate	439.6 ± 0.2
Glucose	97.6 ± 0.2
Succinate	12.3 ± 0.35
Acetate-glucose	84.7 ± 0.35
Acetate-succinate	17 ± 0.5
Palmitate-glucose	217.5 ± 0.51
Palmitate-succinate	≤5
Middlebrook medium	120.5 ± 0.5

[a] Cell extracts were made from cultures grown on the indicated carbon source and assayed for enzyme activity. The values represent the mean of at least three independent experiments.

couple of hours on ice. Neither the addition of glycerol nor bovine serum albumin improved its stability.

Effects of carbon sources on Icl and AceA induction. In most organisms, the expression of isocitrate lyase activity is dependent on the carbon source in the growth medium. To elucidate whether the induction of Icl and AceA expression and activity in *M. avium* and *M. tuberculosis* is also dependent on nutrients, crude bacterial extracts were assayed for both isocitrate lyase activity and expression.

The specific total activity of the isocitrate lyase in *M. avium* varied widely depending on the primary carbon source (Table 1). The lowest level of induction was observed when cells were grown on succinate or glucose, with specific activities of 12.3 ± 0.35 and 97.6 ± 0.2 nmol/min/mg of protein, respectively. Growth on acetate or on palmitate led to high levels of induction (439.6 ± 0.2 and 1,193.4 ± 0.2 nmol/min/mg of protein, respectively). We also determined whether either acetate or palmitate could still upregulate isocitrate lyase activity when the cultures were given an alternate carbon source. *M. avium* cells were harvested in mid-log phase to avoid depletion of any of the carbon sources. When cultures were grown on palmitate with glucose, the levels of enzyme activity were about half that of acetate-grown cultures (217.5 ± 0.51 nmol/min/mg of protein). Less activity was demonstrated with *M. avium* cultivated on acetate plus glucose at 84.7 ± 0.35 nmol/min/mg of protein. Growth of *M. avium* on a combination of succinate and acetate or on succinate and palmitate in minimal medium, however, did not result in any upregulation of the enzyme. Thus, the presence of an alternate carbon source does not completely repress induction of the isocitrate lyase unless succinate is present. Because the total isocitrate lyase activity is the product of two discrete enzymes, we examined the relative levels of Icl and AceA protein by immunoblotting comparable amounts of SDS-PAGE separated extracts from bacilli grown under differing conditions (Fig. 3). It was found that the activity levels correlated with the relative amount of Icl protein detected in immunoblots. Expression of AceA in *M. avium* is apparently only upregulated by acetate and not by palmitate, while succinate, again, repressed expression of this enzyme completely. The data indicate strongly that the primary regulation of carbon flux into the glyoxylate pathway is at the level of isocitrate lyase expression. The pronounced effect of succinate on the Icl and AceA production is likely attributable to feedback inhibition since succinate is one of the products of isocitrate lyase activity.

We also examined the levels of expression of Icl and AceA in *M. tuberculosis* CSU93 and $H_{37}R_v$ under different culture conditions. However, *M. tuberculosis* is more fastidious than *M. avium* and would not grow on minimal medium that was not supplemented with Casamino Acids in addition to acetate or palmitate, although the latter carbon sources were limiting for growth. As a result we do not know whether the activity levels obtained were maximal. By according to glucose-grown bacteria an arbitrary value, we found that palmitate induced isocitrate lyase activity (threefold) to a greater extent than acetate (twofold) and that succinate repressed the enzyme activity below levels of detection. Glucose, on the other hand, could not repress expression of activity when given together with acetate or palmitate (these remained two- to threefold). Once again these data were substantiated by immunoblots with anti-Icl and anti-AceA antibodies (Fig. 3). In contrast to *M. avium*, AceA production in strain CSU93 increased when the cells were grown on palmitate. Interestingly, when grown under the same conditions, only strain CSU93 and not $H_{37}R_v$ expressed levels of AceA detectable by immunoblot (not shown). The apparent lack of expression of this enzyme in $H_{37}R_v$ may indicate that the ORF(s) Rv1915 and Rv1916 do not give rise to a product.

Our attempts to measure malate synthase activity in crude mycobacterial extracts were unsuccessful. However, preliminary studies with an antipeptide antibody against the malate synthase from *M. tuberculosis* CSU93 showed that under the conditions examined no differential expression of protein could be observed (not shown).

DISCUSSION

Isocitrate lyase and the glyoxylate bypass are required by microorganisms for growth on the two-carbon compound acetate. In addition, many fatty acids are metabolized, at least partially, to acetyl-CoA, which is mainly catabolized via the TCA cycle. However, with each turn of the cycle two carbon atoms are lost as CO_2. The glyoxylate pathway circumvents the loss of carbon dioxide and so facilitates a net gain of carbon into cellular structures. It acts as a link in the formation of carbohydrates from fatty acids and occurs in seedlings rich in oil such as castor bean (*Ricinus communis*) (16). It is therefore not surprising that the enzymes of this cycle are present in mycobacteria, which are renowned for their lipid content.

By searching the mycobacterial databases with the amino terminus of a protein which is abundant in intracellular *M. avium*, we were able to align the sequence with an ORF (Rv0467, named *icl*) which showed high similarity to an isocitrate lyase of *C. glutamicum* and *R. fascians* (28, 38). The latter organisms, as well as mycobacteria, belong to the *Actinomycetes*, and high similarity between equivalent proteins of these groups is not unusual (13, 14). Interestingly, the mycobacterial databases contain another ORF (*aceA*) which shows some homology to isocitrate lyases. In $H_{37}R_v$ this ORF contains two genes, *aceAa* (Rv1915) and *aceAb* (Rv1916). The two genes overlap by one nucleotide according to the published database of the Sanger Center. This arrangement is in contrast to the highly similar ORF in CSU93 (MT1966; TIGR database), as well as a closely related ORF from *M. leprae* (7), which both show a single, continuous reading frame.

Polyclonal antibodies, generated against both a synthetic peptide from the predicted amino acid sequence of AceA and the recombinant protein itself, indicated that the enzyme is expressed as a protein of approximately 67 kDa in *M. tuberculosis* CSU93 under the same conditions as Icl. In $H_{37}R_v$, however, this gene appears to be a pseudogene, since AceA is not expressed. The high K_m value of the purified, recombinant enzyme (1.3 mM versus 145 μM for Icl) and its lower V_{max}

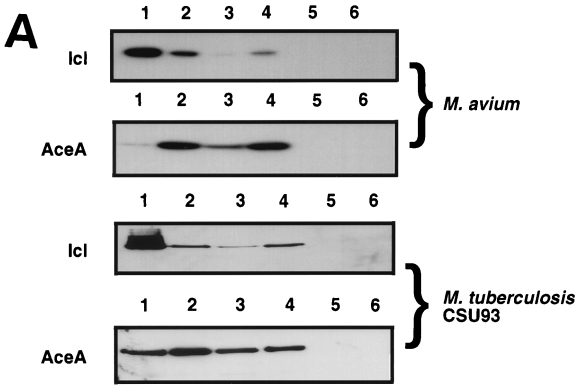
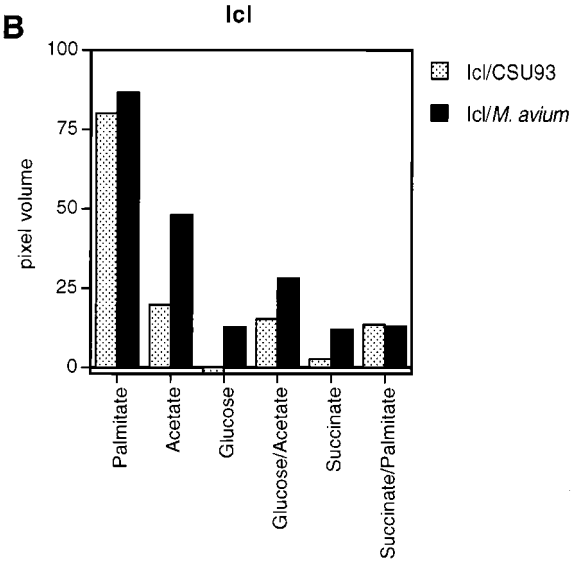
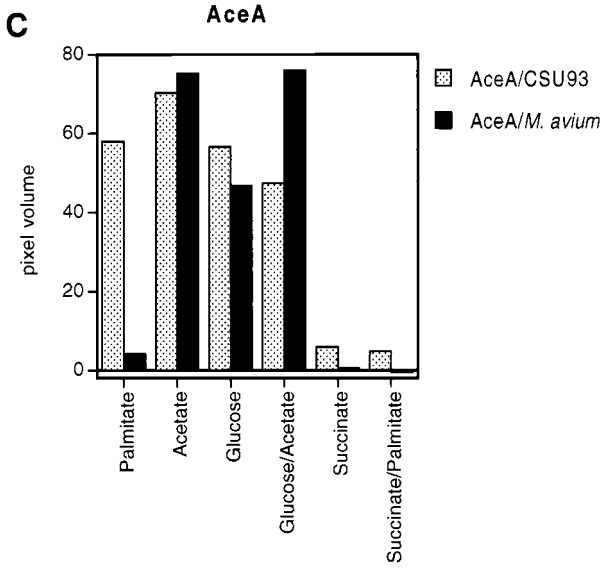


FIG. 3. Effects of carbon source on expression of Icl and AceA in *M. avium* and *M. tuberculosis*. The mycobacterial cultures were grown in minimal medium with the carbon sources indicated. Cells were harvested, and cell extracts were prepared. A total of 2 μg of total bacterial extract was applied to each lane. Proteins were transferred to a nitrocellulose membrane. (A) The extracts were probed with either an anti-Icl peptide antibody or an anti-AceA antibody raised against the recombinant protein. Antibodies were diluted 1:10,000, respectively. Lanes: 1, minimal medium (MM) plus palmitate; 2, MM plus acetate; 3, MM plus glucose; 4, MM plus glucose and acetate; 5 MM plus succinate; 6, MM plus succinate and palmitate. Quantification of the Icl (B) and AceA (C) amounts. All membranes were exposed to Amersham Hyperfilm MP, and the volumes (i.e., the sum of pixel values within an object) were quantitated by densitometry (FluorImager SI; Molecular Dynamics). The carbon sources used for each culture and the respective strains are indicated.





(0.45 versus 1.3 μmol/min/mg), together with its absence in $H_{37}R_v$, lead us to speculate that the protein might assume a subordinate role in *M. tuberculosis*. AceA may only be needed when the level of isocitrate rises above a certain threshold. The intracellular concentration of isocitrate has not been calculated for *Mycobacterium* spp.; however, in *Pseudomonas nautica* the intracellular isocitrate concentration did not exceed 300 μM (31), which (if applicable for *M. tuberculosis*) is considerably below the K_m for AceA. Since the K_m for threo D-(s) isocitrate in the cytoplasmic extracts of *M. avium* grown on palmitate corresponds to the K_m for the recombinant, purified Icl, we conclude that under normal conditions AceA does not play a major role in the glyoxylate pathway of mycobacteria.

Affinity-purified, antipeptide antibody directed against the Icl protein, in conjunction with enzyme assays, demonstrated that Icl expression can be upregulated by the growth of both *M. avium* and *M. tuberculosis* in minimal medium supplemented with either acetate or palmitate. However, a low level of Icl expression was consistently observed irrespective of the carbon source. This was more marked in *M. tuberculosis*, where the Icl appeared to be particularly responsive to oxygen availability since cultures grown under aeration produced less enzyme than comparable cultures grown for 1 to 2 weeks without disturbance (not shown). This correlates with the observations of Wayne et al. (41), who described a transient increase of isocitrate lyase activity in *M. tuberculosis* cells during adaptation to microaerophilic conditions. Our analysis also reveals that isocitrate lyase activity is elevated in bacteria when grown on other carbon sources in the presence palmitate and acetate. A similar situation has been described for *C. glutamicum*, where isocitrate lyase activity is also high when the organism is grown on various carbon sources together with acetate. This contrasts with other microorganisms, such as *E. coli* (17), *Rhodobacter capsulatus* (1), and *Bradyrhyzobium japonicum* (10), that express isocitrate lyase when acetate or fatty acids provide the sole carbon source but do not express the enzyme in cultures grown on any other substrate, irrespective of the presence or absence of acetate.

Obviously the arrangement of the genes of the glyoxylate shunt pathway within the genomes of organisms can have a fundamental influence on the regulation and routing of carbon flux. In *E. coli*, the isocitrate lyase gene *aceA* and the malate synthase A gene *aceB* are organized as an operon together with *aceK* (isocitrate dehydrogenase kinase-phosphatase [IDHP]) (5). IDHP activates and deactivates isocitrate dehydrogenase by phosphorylation and dephosphorylation, thereby regulating the flux of isocitrate into the glyoxylate cycle (20, 21). A repressor protein, IclR (24) that in turn is positively regulated by FadR, a fatty acid degradation-synthesis regulator (11), regulates the expression of this operon. This cascade of regulation limits expression of the *ace* operon to growth on acetate and fatty acids as the sole carbon source. If another preferred substrate is present (e.g., glucose or pyruvate), expression of the glyoxylate bypass enzymes is repressed (6, 18).

Analyses of the databases for *M. tuberculosis* $H_{37}R_v$ and CSU93 reveal another arrangement: the genes encoding malate synthase and isocitrate lyase are separated by several thousand base pairs (4). The malate synthase gene shows very high homology to the corresponding *C. glutamicum* enzyme (29). However, both of these genes show weak identity with *aceB*, which encodes malate synthase A from *E. coli*, which is associated with the glyoxylate cycle. In contrast, the malate synthase genes of both *M. tuberculosis* and *C. glutamicum* are much more closely related (>55% identity) to *glcB*, which encodes malate synthase G, part of the glycolate utilization pathway of *E. coli* (27).

While the sequence similarity between the enzymes of the glyoxylate cycle of *M. tuberculosis* and *C. glutamicum* is very high, the gene organization appears to be quite different, suggesting that the cycle may respond to different regulatory pathways. In *M. avium*, the isocitrate lyase is clearly upregulated when the cells utilize acetate as the sole carbon source. Growth on palmitate increases this enzyme activity an additional twofold. Furthermore, the amount of Icl expressed was elevated when *M. avium* were grown intracellularly. However, it should be noted that we found the levels of expression of Icl by intracellular *M. avium* to be extremely variable, and we suspect that the intracellular environment per se was only part of the stimulus for Icl upregulation. In a recent series of experiments conducted by Graham and Clark-Curtiss employing differential gene expression, these authors also detected the expression of Icl but not AceA in intracellular *M. tuberculosis* $H_{37}R_v$ (9). One might speculate that the intracellular environment of *M. avium* and *M. tuberculosis*, the mycobacterium-containing phagosome, is one of restricted nutrients and that host lipids and other long-chain fatty acids may be one of the more abundant carbon sources. This, in combination with a microaerophilic environment, could lead to an upregulation of the glyoxylate cycle as an anapleurotic sequence for replenishing the pool of substrates used for biosynthetic processes. Our current studies are aimed at delineating the environmental stimuli responsible for mobilization of the glyoxylate pathway.

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