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Site-Directed Mutagenesis of Lysine 193 in *Escherichia coli* Isocitrate Lyase by Use of Unique Restriction Enzyme Site Elimination

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By a newly developed double-stranded mutagenesis technique, histidine (H), glutamate (E), arginine (R) and leucine (L) have been substituted for the lysyl 193 residue (K-193) in isocitrate lyase from *Escherichia coli*. The substitutions for this residue, which is present in a highly conserved, cationic region, significantly affect both the K_m for D_s-isocitrate and the apparent k_{cat} of isocitrate lyase. Specifically, the conservative substitutions, K-193→H (K193H) and K193R, reduce catalytic activity by ca. 50- and 14-fold, respectively, and the nonconservative changes, K193E and K193L, result in assembled tetrameric protein that is completely inactive. The K193H and K193R mutations also increase the K_m of the enzyme by five- and twofold, respectively. These results indicate that the cationic and/or acid-base character of K193 is essential for isocitrate lyase activity. In addition to the noted effects on enzyme activity, the effects of the mutations on growth of JE10, an *E. coli* strain which does not express isocitrate lyase, were observed. Active isocitrate lyase is necessary for *E. coli* to grow on acetate as the sole carbon source. It was found that a mutation affecting the activity of isocitrate lyase similarly affects the growth of *E. coli* JE10 on acetate when the mutated plasmid is expressed in this organism. Specifically, the lag time before growth increases over sevenfold and almost twofold for *E. coli* JE10 expressing the K193H and K193R isocitrate lyase variants, respectively. In addition, the rate of growth decreases by almost 40-fold for *E. coli* JE10 cells expressing form K193H and ca. 2-fold for those expressing the K193R variants. Thus, the onset and rate of *E. coli* growth on acetate appears to depend on isocitrate lyase activity.

The glyoxylate cycle, which functions primarily in oil-rich seedlings, fungi, and microbes, diverts isocitrate from the carbon-utilizing steps of the tricarboxylic acid cycle, thereby enabling the anabolic conversion of acetyl coenzyme A into metabolic intermediates necessary for growth and for the formation of carbohydrates from fatty acids. Isocitrate lyase and malate synthase are the two key enzymes which enable this conversion. Isocitrate lyase, the first enzyme in the branch to the glyoxylate cycle, competes with isocitrate dehydrogenase for D_s-isocitrate. Isocitrate lyase has been found in many oil-rich seedlings of more highly evolved plants, in fungi, bacteria, and parasitic invertebrates, and even in some insects (for a review, see reference 26). In most cases, it is tetrameric and requires Mg^{2+} or Mn^{2+} and either a thiol or EDTA for activity. The catalytic mechanism probably involves acid-base catalysis, in which isocitrate is deprotonated by the enzyme, leading to glyoxylate and succinate anion, which is then protonated to succinate to complete the reaction (26). The 192-kDa *Escherichia coli* enzyme is approximately three-fourths the size of the isocitrate lyase of more highly evolved plants and seems to require phosphorylation of a histidine for activity (23).

To date, the gene or cDNA for isocitrate lyase has been cloned and sequenced from rapeseed (5), cotton (25), castor bean (3), *Candida tropicalis* (2), *Saccharomyces cerevisiae* (10), *Emericella nidulans* and *Neurospora crassa* (11), and *E. coli* (18, 22). Alignment of the derived protein sequences reveals four highly conserved regions. The first and largest region, from amino acid 177 through 200 in the *E. coli* enzyme, contains two lysine and two histidine residues (see Discussion). This region is of particular interest, because previous affinity labeling experiments with bromopyruvate have implicated cysteine 195 as an active-site residue (13). Thus, considering the catalytic mechanism for isocitrate lyase (26) and the highly anionic character of the tricarboxylic substrate, it is plausible to postulate an important binding and/or catalytic function for these conserved basic residues. With this in mind, the present study considers the importance of the first conserved lysine residue (K-193) in this region.

MATERIALS AND METHODS

Media, enzymes, and reagents. Luria-Bertani (LB) medium and minimal M9 medium were made according to the protocol described by Sambrook et al. (24). Medium was solidified for petri plates by using 1.5% (wt/vol) agar (Difco). Antibiotics were added as necessary.

Restriction endonucleases were obtained from GIBCO BRL (Life Technologies Inc.) and used as recommended. Except for the T4 5′ DNA kinase, which was obtained from Boehringer Mannheim, the enzymes and buffers used for mutagenesis were supplied with a Transformer kit (Clontech Laboratories), and with the exception of 5′-α-thio[^{35}S]dATP (1,000 to 1,500 Ci/mmol), which was supplied by DuPont NEN Research Products, the enzymes and buffers used for sequencing were supplied with a Sequenase kit (United States Biochemical Corp.).

Bacterial strains and plasmids. All bacterial strains used in this study are derivatives of *E. coli* K-12. *E. coli* BMH-71-18 (*mutS*) was supplied with the Transformer kit (Clontech Laboratories) and was used during mutagenesis of the isocitrate lyase (*aceA*) gene. *E. coli* JE10 (Δ*aceA recA*::Tn*10*),

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derived from JM105 (18), was used to express isocitrate lyase after transformation with the pICL1 plasmid. pICL1 is a derivative of the pDR540 vector (Pharmacia LKB Biotechnology, Piscataway, N.J.), which contains *aceA*, the gene for isocitrate lyase (18).

Deoxyoligonucleotides. With the exception of the 29-base deoxyoligonucleotide to alter the *Ssp*I restriction enzyme site to an *Eco*RV site, which was supplied by Clontech Laboratories, all the deoxyoligonucleotides were synthesized by Gerhard Munske (Washington State University) by using phosphoramidite chemistry. The following four deoxyoligonucleotides were used to introduce the mutations into the K-193 codon of the *aceA* gene in pICL1: K-193→H (K193H), GGCGTCAGTGCATAAATGCGGTC; K193E, GGCGTCAGTGGAGAAATGCGGTC; K193R, GGCGTCAGTGCGGAAATGCGGTC; and K193L, GGCGTCAGTGCTGAAATGCGGTC. For sequencing, the primer CGTTGCCGATGCGGAAGC was used. This primer enables the addition of ca. 100 bp 5′ to the K-193 codon.

Mutagenesis of the *aceA* gene. Mutagenesis was carried out by the unique restriction enzyme site elimination technique (7) using the Transformer mutagenesis kit (Clontech Laboratories). In a 20-μl volume, ca. 0.1 μg of the pICL1 plasmid (18), 0.1 μg of the *Ssp*I-to-*Eco*RV selection deoxyoligonucleotide, and 0.1 μg of one of the four K-193 mutagenic deoxyoligonucleotides were suspended in annealing buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl; pH 7.5; 4°C), and the mixture was incubated at 100°C for 3 min and then placed on ice for 5 min. Then, 3 μl of synthesis buffer (a component of the Transformer kit), which contained the deoxyoligonucleotides in Tris-HCl buffer, pH 7.5, was added to the mixture along with 3 U of T4 DNA polymerase and 4 U of T4 DNA ligase, and the volume was adjusted to 30 μl. After incubation at 37°C for 2 h, the synthesis-and-ligation reaction was stopped by adding 3 μl of stop solution (0.25% sodium dodecyl sulfate [SDS]–5 mM EDTA, pH 8.0) and incubating the solution at 65°C for 5 min. At this point, 15 μl of the mutagenesis reaction mixture was used to transform 300 μl of competent *E. coli* BMH-71-18. These cells were made competent by using a RbCl-$MnCl_2$-$CaCl_2$ solution according to the procedures described by Hanahan (12). Transformation of these cells was accomplished by incubating the competent cells with DNA from the mutagenesis reaction for 40 min on ice and incubating the cell-DNA mixture at 42°C for 45 s and then adding 1 ml of LB medium to the mixture and incubating the mixture at 37°C with shaking for 1 to 2 h. Following the LB medium incubation, 4 ml of LB medium containing 100 μg of ampicillin per ml was added to the cells, and the liquid culture was incubated overnight at 37°C. The following day, plasmid DNA was isolated from these cultures by the cetyltrimethylammonium bromide precipitation technique (6). Plasmid DNA from the culture was resuspended in 25 μl of 10 mM Tris–1 mM EDTA, pH 8.0, at 4°C. For restriction enzyme digestion, 7 μl of the resuspended DNA was incubated with 13 μl of buffer containing 15 U of restriction enzyme *Ssp*I. After the restriction enzyme reaction mixture was incubated at 65°C for 10 min following a 3-h incubation at 37°C, 300 μl of *E. coli* JE10, a strain that lacks the endogenous isocitrate lyase gene (18), was made competent by the procedure described above. These cells were transformed with 15 μl of the restriction enzyme digest. The transformation procedure was the same as that described above, except that, after incubating in LB medium at 37°C for 1 to 2 h, aliquots of the cells were spread onto plates containing LB medium supplemented with 100 μg of ampicillin and 20 μg of tetracycline per ml. After incubating overnight at 37°C, colonies appearing on these plates were transferred to liquid medium and grown. Then, plasmid DNA was isolated and used for double-stranded dideoxynucleotide sequencing to identify mutants. Typically, the sequencing gels could be easily interpreted from ca. 50 bp upstream of the mutations to ca. 200 bp in the 3′ direction from the mutations.

Isolation of a high-speed supernatant fraction from *E. coli*. An overnight 2-ml culture of *E. coli* JE10 expressing the pICL1 plasmid containing either the wild-type or the mutant form of *aceA* was added to 200 ml of LB medium with 20 μg of tetracycline and 150 μg of ampicillin per ml. After this culture had been incubated at 37°C for 3 h with shaking, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression of isocitrate lyase (18), and this culture was incubated at 37°C for another 2 h. After this second incubation, cells were harvested by centrifugation, and the pellet, which consisted of approximately 1 g of cells, was lysed on ice by resuspending the pellet in 3 ml of lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl; pH 8.0; 4°C), adding 0.08 μg of lysozyme, and then freezing the mixture at −70°C (17). Following freezing, the suspension was slowly thawed at 4°C with intermittent agitation. After thawing, the suspension was placed in a 37°C water bath for 3 to 5 min to ensure complete thawing, and 0.1 mg of DNase was added at ambient temperature. Finally, 10 ml of isocitrate lyase assay buffer (0.05 M MOPS [morpholinepropanesulfonic acid], 5 mM $MgCl_2$, 1 mM dithiothreitol; pH 7.0) was added, and the mixture was centrifuged at 220,000 × *g* (49,000 rpm in a Sorvall Ti 70.1 rotor) for 1 h at 2°C. The supernatant from this high-speed spin was either assayed immediately or stored at −70°C until needed. The protein concentration of this supernatant was determined by the Bradford method (4) using the Bio-Rad protein assay. A solution of bovine serum albumin ranging from 0.2 to 1.0 mg/ml was used as a protein standard.

The high-speed supernatant fraction was also analyzed by nondenaturing polyacrylamide gel electrophoresis (PAGE) (20, 21), polyacrylamide gradient gel electrophoresis (16), and SDS–10% PAGE (15).

Isocitrate lyase assay. The discontinuous method described by Matsuoka and McFadden (18) and Ko et al. (14), with a few modifications, was used to measure the conversion of isocitrate to succinate and glyoxylate. This method exploits a colorimetric reaction between the phenylhydrazone of glyoxylate and ferricyanide (19). Briefly, a 0.95-ml volume of isocitrate lyase assay buffer (0.05 M MOPS, 5 mM $MgCl_2$, 1 mM dithiothreitol; pH 7.0) with 0.1% phenylhydrazine and an aliquot of the high-speed supernatant isolated as described above was equilibrated to 37°C. D_s-isocitrate (50 μl) at 37°C was then added to the reaction mixture as specified. Incubation of the reaction mixture continued for 3 min at 37°C and was stopped with the addition of 450 μl of concentrated HCl. Then, at ambient temperature, 2 ml of H_2O was added, and 50 μl of a 10% solution of potassium ferricyanide was added to initiate color development. After incubation at room temperature for 12 to 15 min, the A_{520} of the reaction mixture was determined. A molar absorption coefficient of 2.6×10^4 for glyoxylate was typically obtained.

Growth of *E. coli* in minimal medium. LB medium cultures of *E. coli* JE10 harboring various forms of pICL1 were diluted to an optical density (OD) of 0.05 with minimal M9 medium. Then 0.5-ml samples of the cultures were added to sterile culture tubes containing 6 ml of minimal M9 medium supplemented with 0.2% glucose or acetate. These cultures

were incubated at 37°C with shaking. The ODs of the cultures at 600 nm (OD_{600}) were determined periodically during their incubations.

Alignment of isocitrate lyase sequences. By using the programs from the Genetics Computer Group sequence analysis software package (8), sequences of isocitrate lyase from eight sources were aligned. This package of programs was provided by the Visualization, Analysis and Design in the Molecular Sciences (VADMS) center at Washington State University. For the alignment, the Pileup program was used primarily. This program uses a simplified version of a progressive pairwise alignment algorithm (9).

RESULTS

The lysine residue at position 193 of isocitrate lyase from *E. coli* was changed to a histidine, arginine, glutamate, or leucine by mutagenesis of the *aceA* gene in plasmid pICL1 (18) by the newly developed unique restriction enzyme site elimination technique described by Deng and Nickoloff (7). This technique enables directed mutagenesis of double-stranded DNA. Thus, this procedure allows a gene to be altered directly in the expression vector and avoids the time-consuming step of subcloning the gene into a vector capable of single-stranded DNA expression that is necessary with the currently popular site-directed mutagenesis procedures. To our knowledge, this is the first time this technique has been used to investigate the effects of amino acid substitutions on catalysis by an enzyme. Essentially, this is a two-primer technique, in which one mutageneic primer codes for the desired mutation and the other primer destroys a unique restriction enzyme site in the plasmid. In the present case for the *aceA* gene, one primer altered the codon for K-193 and the other primer converted the restriction enzyme *Ssp*I recognition sequence at bp 5630 of pICL1 to an *Eco*RV recognition site (Fig. 1). After alkali denaturation of the pICL1 plasmid, primer annealing and in vitro synthesis using T4 DNA polymerase, mismatch repair-deficient cells of *E. coli* BMH-71-18 were transformed with the reaction mixture. Plasmid DNA was isolated from cultures of the transformed *E. coli* grown overnight. The isolated plasmid DNA was then digested with *Ssp*I to linearize nonmutated plasmids, since linear DNA transforms much less efficiently than closed circular DNA. Finally, *E. coli* JE10 cells, which lack the isocitrate lyase gene *aceA* (18), were transformed with the *Ssp*I digestion mixture. These cells were then grown on plates containing LB medium, and the plasmid DNA isolated from the colonies which developed on these plates was sequenced to identify mutants (Fig. 2).

In this case, the unique site elimination mutagenesis technique worked well and resulted in eight mutants of the 12 colonies chosen for sequencing. Specifically, 12 colonies, 3 for each of the four putative K-193 mutations, were selected. By DNA sequencing, it was determined that three K193H, two of three K193R, two of three K193L, and one of three K193E conversions were successful. To verify that the unique site elimination technique had been responsible for the high efficiency of mutagenesis, plasmid DNA that was isolated from the colonies was subjected to restriction enzyme digestion with *Eco*RV (Fig. 3). Of the eight successful mutants identified by sequencing, one of the K193R isolates resulted in anomalous digestion, which was possibly the result of a mixed population of wild-type and mutant DNAs (Fig. 3, lane 9). Indeed, cell lysates prepared from cultures of this isolate resulted in elevated (wild-type-like) enzyme activity, whereas the sequencing gel lanes for this isolate indicated a mutant enzyme. Also, the second K193H isolate did not seem to contain the *Eco*RV site; however, enzyme assays and sequencing both indicated that this isolate contained the desired mutation within the *aceA* gene. In all other cases, isolates which were mutated, as determined by enzyme assays and sequencing, also gained an extra *Eco*RV site while wild-type isolates did not. This cosegregation strongly supports the idea that the double-primer unique restriction enzyme site elimination technique was responsible for the high efficiency of mutations obtained.

After the mutations were verified, 200-ml cultures containing *E. coli* JE10 cells expressing mutant isocitrate lyase were lysed and ultracentrifuged. In addition to high-speed supernatants from wild-type and mutant cells, that from a culture of *E. coli* JE10 containing a modified pICL1 plasmid which has a 400-bp deletion between the two *Dsa*I sites in the *aceA* gene was also prepared to ensure that it was indeed isocitrate lyase activity that was being measured by the assay. *E. coli* JE10 cells harboring this plasmid do not contain a significant amount of enzyme as determined by SDS-PAGE (Fig. 4A and B, lanes 1) and enzyme assay (Table 1). Aliquots of the high-speed supernatant were subjected to SDS-PAGE, which showed that essentially equal amounts of isocitrate lyase protein were present after expression of the wild-type and mutant plasmids (Fig. 4B). Furthermore, nondenaturing PAGE (Fig. 5) and gradient PAGE (not shown) established that the mutations did not interfere with assembly of the tetrameric subunit complex characteristic of the wild-type enzyme.

The high-speed supernatants were assayed for enzyme activity. Of the four mutated proteins, only the K193H and K193R forms had significant isocitrate lyase activities (Table 1). The K_m value for the K193R mutant was more than twice that for the wild type, and that for the K193H was almost five times higher. Furthermore, the mutations appreciably reduced the specific activity. The specific activity for K193R was ca. 14-fold lower than that for the wild-type enzyme, and the specific activity for K193H was ca. 50-fold lower than that for the wild-type enzyme. In light of the closely similar levels of expression and the assembly of subunits into tetramers in all mutants, these specific activities are proportional to turnover numbers or k_{cat} values.

In addition to the activity assays, attempts to grow *E. coli* JE10 cells, which lack the *aceA* gene, in minimal M9 medium supplemented with acetate after transformation by each pICL1 plasmid modification were made. An active glyoxylate cycle is necessary for growth on acetate, so *E. coli* cells expressing inactive isocitrate lyase should be unable to grow on acetate. Furthermore, since this enzyme is at the branch to the glyoxylate cycle, the growth rate of *E. coli* on acetate may be related to the activity of this enzyme. *E. coli* JE10 expressing any of the K-193 variants or the isocitrate lyase partial deletion was able to grow in minimal M9 medium supplemented with glucose (Fig. 6). However, in acetate medium, only those cultures expressing the wild type or the K193R or K193H variant of the enzyme were able to grow; cells expressing the K193E or K193L protein or the isocitrate lyase partial deletion did not grow (Fig. 6). This result is consistent with the measured activity of the mutant enzymes, since only the K193R and K193H forms had activity in vitro (Table 1). In addition, the growth of JE10 expressing the K193R or K193H mutant enzyme corresponded approximately to the activity of the respective enzyme (Table 1, last column). Specifically, the lag times for *E. coli* cells expressing these forms of isocitrate lyase were significantly longer than that for cells expressing the wild-

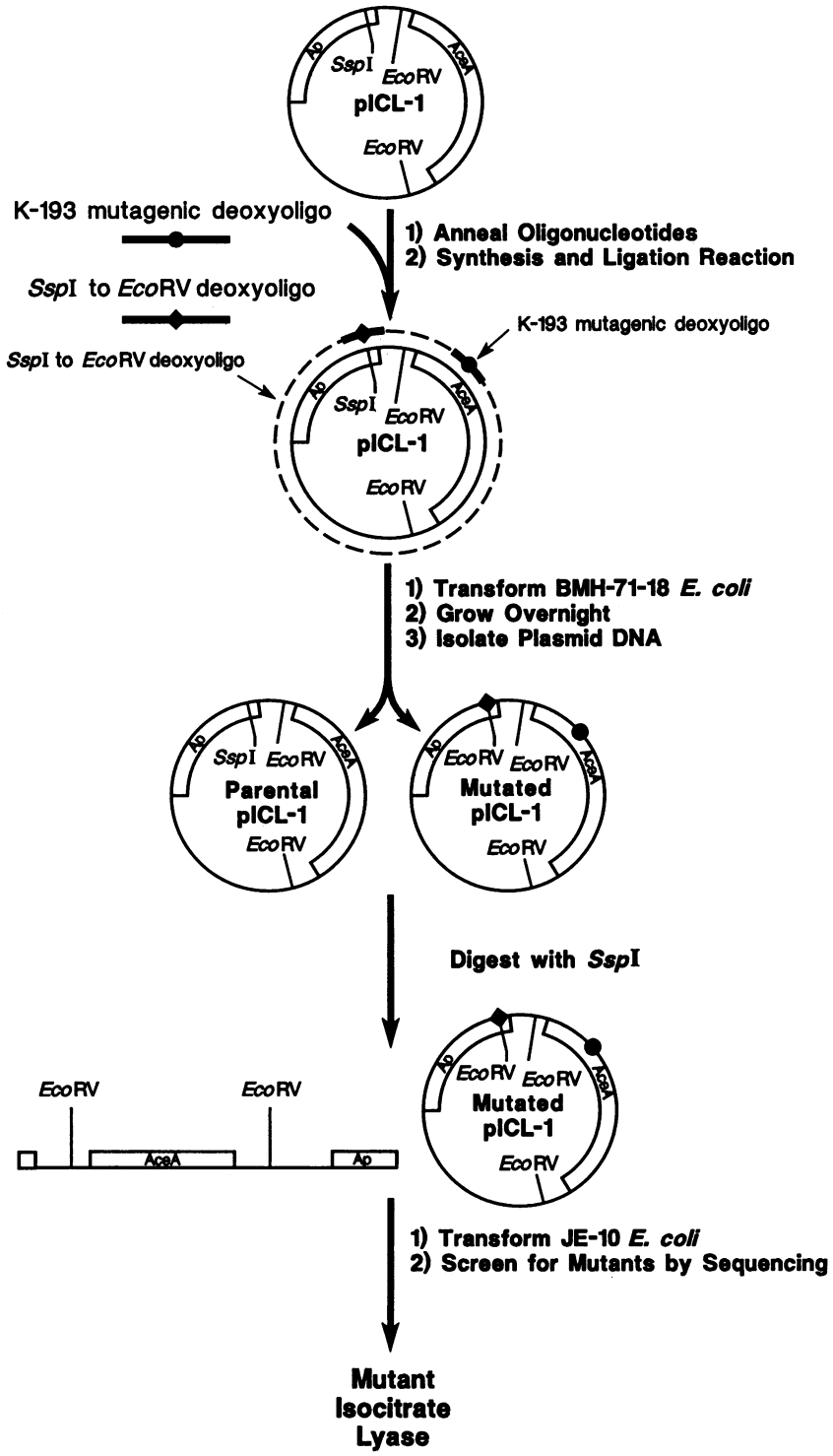

FIG. 1. Unique restriction enzyme site elimination mutagenesis. The K-193 residue of the *aceA* gene in plasmid pICL1 was altered by the unique restriction enzyme site elimination technique. This is a two-primer technique in which one mutagenic primer codes for the desired mutation and the other primer destroys a unique restriction enzyme site in the plasmid (in this case, the *Ssp*I site in pICL1). After *Ssp*I digestion, *E. coli* JE10 cells, which lack the isocitrate lyase gene *aceA* (18), were transformed with the *Ssp*I digestion mixture. These cells were then grown on plates containing LB medium, and plasmid DNAs isolated from the colonies which developed on these plates were sequenced to identify mutants.

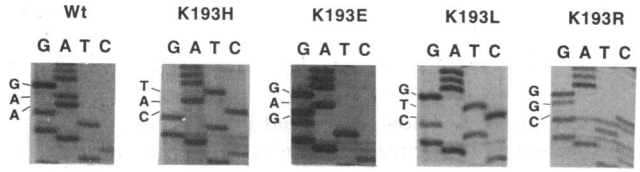

FIG. 2. Sequencing gel showing K-193 mutations of the *aceA* gene. Dideoxynucleotide sequencing was used to verify that the bases in wild-type *aceA* which encode the lysine 193 (AAG) in isocitrate lyase were replaced by those for histidine (CAT), glutamine (GAG), arginine (GGC), or leucine (GTC).

type enzyme. As opposed to the 12.5 h which elapsed before *E. coli* JE10 expressing the wild-type isocitrate lyase began logarithmic growth, cells expressing the K193R form or the K193H form had lag phases of 22 and 87 h, respectively (Fig. 6). Furthermore, the growth rates of *E. coli* JE10 expressing the K193R and K193H forms of isocitrate lyase were reduced. The OD_{600} during logarithmic growth for the culture expressing the wild-type enzyme increased at a rate of 0.08 OD units per h. This rate can be compared with 0.04 OD units per h for the culture expressing the K193R enzyme and 0.003 OD units per h for the culture expressing K193H isocitrate lyase. *E. coli* JE10 expressing any form of isocitrate lyase grew at a rate of 0.16 OD units per h in minimal M9 glucose medium (Fig. 6).

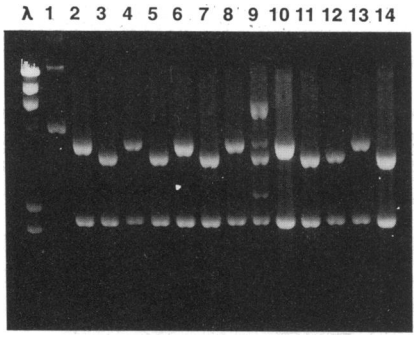

FIG. 3. Restriction enzyme *Eco*RV digest of plasmids mutated in the *aceA* gene. Plasmid DNAs which were isolated from colonies that developed after *E. coli* JE10 cells were transformed with the *Ssp*I restriction enzyme reaction mixtures were digested with *Eco*RV. The agarose gel shows the results of these digests. This digestion determined whether an additional *Eco*RV site occurred coincidentally with a successful mutagenesis of *aceA*, as would be expected on the basis of the mutagenesis protocol. Whereas *Eco*RV digestion of the wild-type pICL1 results in two bands, 3,704 and 2,114 bp (lane 2), digestion of a pICL1 plasmid which has an *Eco*RV site substituted for the unique *Ssp*I site will produce three fragments of 3,329, 2,114, and 375 bp. This will be reflected in the presence of a fragment of 3,329 instead of 3,704 bp and simply result in a smaller upper band (i.e., 3,329 instead of 3,704 bp) in each digest (e.g., compare lanes 2 [wild type] and 3 [a plasmid with an extra *Eco*RV site]). The smallest restriction fragment (375 bp) has been run out of the gel slab. Lane λ, λ phage DNA digested with *Hin*dIII for a size standard; lanes 1 and 2, wild-type pICL1 that was undigested or digested with *Eco*RV, respectively; lanes 3 to 5, pICL1 plasmids from the K193H isolates digested with *Eco*RV; lanes 6 to 8, plasmid DNAs from K193E isolates digested with *Eco*RV; lanes 9 to 11, digested K193R plasmid DNA preparations; lanes 12 to 14, digested K193L plasmid DNA preparations.

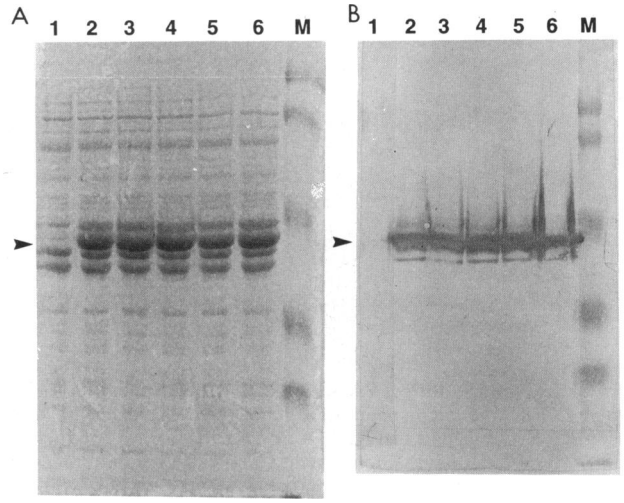

FIG. 4. SDS-PAGE and Western blot (immunoblot) of high-speed supernatant. The high-speed supernatant, derived by ultracentrifugation of lysed cells from a 200-ml culture resuspended in isocitrate lyase assay buffer was prepared from cultures expressing each of the enzyme variants. (A) An SDS gel which had been polymerized from 10% acrylamide and stained with Coomassie blue after electrophoresis. Lane M, 106-, 80-, 49.5-, 32.5-, 27.5-, and 18.5-kDa size standards; lanes 2 to 6, aliquot portions of the high-speed supernatants containing 20 μg of protein each from cultures expressing the wild-type, K193H, K193E, K193R, and K193L forms of isocitrate lyase, respectively; lane 1, culture harboring a pICL1 deletion mutant in which the *aceA* gene is missing 400 bp between the restriction enzyme *Dsa*I sites and, thus, is not expressed. (B) Western blot of a gel run under the same conditions as the gel shown in panel A. The position of the subunit of isocitrate lyase is indicated (arrowhead). After transfer of protein from the gel to the membrane, the membrane was developed by using antibodies against isocitrate lyase raised in rabbits.

DISCUSSION

This article describes mutagenesis to produce the first mutant forms of isocitrate lyase, an enzyme located after the point at which isocitrate branches into either the glyoxylate cycle or tricarboxylic acid cycle. Lysine 193 in this enzyme from *E. coli* was chosen for mutagenesis because this residue lies in a highly conserved cluster of amino acid residues (Fig. 7). The corresponding residue is present in the eight known isocitrate lyase sequences of bacteria, fungi, and the more highly evolved plants. Furthermore, previous bromopyruvate labeling studies by Ko and McFadden (13) have shown that alkylation of cysteine 195 (C-195) inactivates isocitrate lyase from *E. coli* in a manner consistent with saturation kinetics and that either glyoxylate or isocitrate protects

TABLE 1. Kinetic constants for isocitrate lyase variants

Enzyme	K_m (mM)	Sp act[a]	Relative activity
Wild type	0.26 ± 0.04	11.8 ± 3.7	100
Deletion mutant	ND[b]	0	0
K193H	1.1 ± 0.4	0.24 ± 0.04	2
K193E	ND	0	0
K193R	0.69 ± 0.1	0.84 ± 0.2	7
K193L	ND	0	0

[a] In micromoles of glyoxylate per minute per milligram of protein. Specific activities of less than 8.5×10^{-4} μmol/min/mg are given as 0.
[b] ND, not determined.

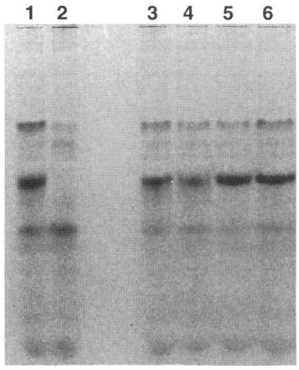

FIG. 5. Nondenaturing PAGE of high-speed supernatant. The aliquots from the high-speed supernatant are the same as those described in the legend to Fig. 4. A nondenaturing gel polymerized from 7% acrylamide was stained with Coomassie blue after electrophoresis. Lane 1, high-speed supernatant containing the wild-type enzyme; lane 2, fraction from cells harboring the pICL1 plasmid with the isocitrate lyase deletion described in the legend to Fig. 4; lanes 3 to 6, aliquots of the high-speed supernatants containing 20 μg of protein each from cultures expressing the K193H, K193E, K193R, and K193L forms of isocitrate lyase, respectively. The band for tetrameric isocitrate lyase is indicated (arrowhead). Nondenaturing gradient PAGE with gels polymerized from 4 to 30% acrylamide demonstrated that the native isocitrate lyase proteins have molecular weights that are the same as that of the wild type.

against this alkylation. In addition, mutagenesis of C-195 to S-195 results in a completely inactive but assembled enzyme (unpublished data). These findings and the alignment shown (Fig. 7) strongly indicate that the conserved amino acid residues from 177 through 200 in the *E. coli* enzyme are part of the active-site region of the enzyme. Since the tricarboxylic substrate, isocitrate, is anionic and the proposed reaction mechanism requires the participation of a base (26), it was reasonable to postulate at the outset of this study that one or more of the two lysine and two histidine residues play a significant role in substrate binding and/or catalysis. In the present study, one of these residues, lysine 193 has been replaced by site-directed mutagenesis with histidine, arginine, leucine, or glutamate. Of the resulting four variants of isocitrate lyase, only K193H and K193R retain some activity. Both enzymes show somewhat elevated K_m values for the substrate D_s-isocitrate. However, the decrease in the turnover number for both mutants is much more striking. The k_{cat}/K_m ratios are 0.0053 and 0.028 for the K193H and K193R variants, respectively, in comparison with the wild-type-enzyme ratio, when normalized to 1.0. These results suggest that these substitutions substantially affect catalysis more than binding and indicate that K-193 functions primarily as a catalytic residue. Although the substitution of either histidine or arginine for K-193 does not alter the cationic charge in the conjugate acid form, the substituted residues have different pK_a values and different distances between the α-carbon and the charged center. Thus, it is reasonable that both substrate binding and catalytic capacity would be changed by substituting histidine or arginine for lysine at position 193. Since the K193E and K193L forms are inactive, a center that is cationic at the pH of the assay, 7.0, is certainly required.

In addition to determining the relative activities of the various mutant forms of isocitrate lyase, the effects of complementation by these mutant enzymes on the growth of isocitrate lyase-deficient *E. coli* JE10 have been examined. Interestingly, it seems that the activity of isocitrate lyase can be related to growth rate on acetate. *E. coli* JE10 cells expressing mutant forms of isocitrate lyase, which display only a fraction of the activity of the wild-type enzyme, take longer to begin growing and, once logarithmic growth begins, grow at slower rates. Specifically, the lag phase for *E. coli* cultures expressing the K193R variant is twice as long as that of cultures expressing the wild-type enzyme, and subsequently these cultures grow at only half the rate. Moreover, cultures expressing the K193H enzyme, the less active of the two mutant forms of isocitrate lyase, take ninefold longer to start logarithmic growth and grow at 2 to 3% of the rate of the *E. coli* cells expressing the wild-type enzyme. These observations indicate that for *E. coli* JE10, the growth

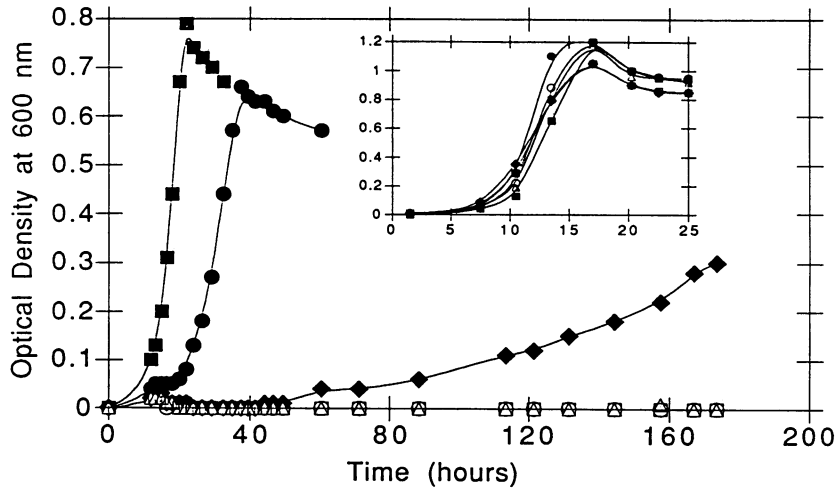

FIG. 6. Growth on glucose and acetate of *E. coli* JE10 expressing isocitrate lyase variants. The main graph shows growth curves for *E. coli* JE10 *aceA* mutants expressing various forms of the enzyme, including the partial isocitrate lyase deletion mutant, in minimal M9 liquid medium supplemented with 0.2% acetate. OD_{600} is plotted against time for JE10 cells expressing either the wild-type isocitrate lyase (■); the K193R (●), K193H (◆), K193E (○), or K193L variant (△); or the deletion mutation (□). The inset shows the results for the same experiment with the exception that the minimal medium was supplemented with 0.2% glucose instead of acetate.

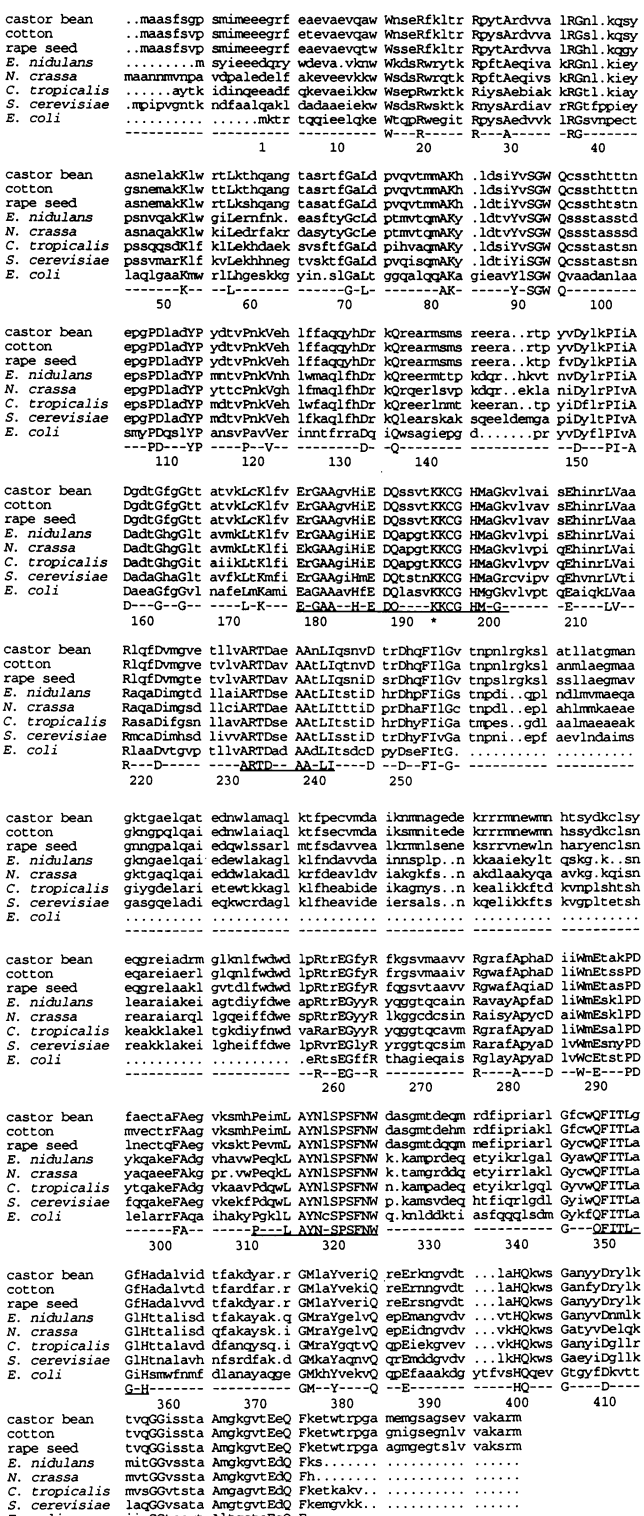

FIG. 7. Alignment of eight isocitrate lyase sequences. The eight known sequences are aligned to show conserved residues (uppercase letters). Dashes in the bottom row represent nonconserved residues. The numbering corresponds to the residues of the *E. coli* isocitrate lyase only. On the basis of additional data (8a), the *E. coli* sequence reported by Matsuoka and McFadden (18) has been corrected from residue 419 to 434 and is now in agreement with the carboxy-terminal sequence of Rieul et al. (22) and the complete sequence of Byrnes (National Biomedical Research Foundation protein data base accession no. S05692). Gaps in isocitrate lyase sequences, needed to properly align highly similar regions from different sequences, are indicated by dots. Four highly conserved regions are underlined. The location of K-193 in the highly conserved stretch between residues 177 and 200 is indicated (*).

rate on acetate is roughly proportional to expression of the isocitrate lyase gene introduced through transformation and that impaired catalysis by mutated enzymes, especially the K193H mutant, severely limits this growth and, presumably, the flux of carbon through the glyoxylate cycle. These results provide direct evidence that reduced isocitrate lyase activity limits the growth rate of *E. coli* on acetate.

The placement of K-193 in the active-site domain of isocitrate lyase from *E. coli* is of special interest because of the recent preparation of crystals of this enzyme which diffract to better than 2 Å (0.2 nm) (1). Elucidation of the crystal structure should provide detailed information about the architecture of the active site and a test of the present placement of K-193 in that domain. It will also greatly enhance the design of other directed-mutagenesis studies.

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