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Isolation and Expression of the *Escherichia coli* Gene Encoding Malate Dehydrogenase

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An oligodeoxynucleotide specific for a pentapeptide sequence corresponding to amino acid residues 32 through 36 of *Escherichia coli* malate dehydrogenase was chemically synthesized and used to identify the *mdh* gene on plasmid pLC32-38 from the Clarke-Carbon recombinant library. Cells transformed with this plasmid exhibited a 10-fold increase in malate dehydrogenase activity. A 1.2-kilobase *Pvu*II fragment which hybridized with the oligodeoxynucleotide probe was subcloned, and the identity of the *mdh* structural gene was confirmed by partial nucleotide sequence analysis. The expression of the *mdh* gene, as measured by both Northern blotting and enzyme assays, was found to vary over a 20-fold range with different culture conditions.

Despite an extensive literature regarding the purification and kinetic analyses of tricarboxylic acid (TCA) cycle enzymes, little is known about the structures and regulation of genes encoding these enzymes. The results of several studies of facultative metabolism in *Escherichia coli* have implied separate controls for subgroups of these enzymes (1, 13, 21), suggesting that expression of the corresponding genes is unlikely to be constitutive or coordinate. This report describes the isolation and a preliminary analysis of the expression of the *E. coli* gene which encodes malate dehydrogenase.

E. coli mutants deficient in malate dehydrogenase have been isolated by several laboratories. The phenotype of these mutants includes an inability to grow on medium with acetate, malate, lactate, or succinate as a carbon source (8, 15), suggesting that malate dehydrogenase is required for oxaloacetate production during gluconeogenesis. In contrast, malate dehydrogenase function is apparently not required for biosynthetic reactions during aerobic or anaerobic growth on glucose, since *mdh* mutants are viable under these conditions (8).

Results of genetic studies suggest that the *mdh* gene is not closely linked with genes encoding other TCA cycle enzymes. Two different linkage map positions have been reported for *mdh*, one at 70.6 min (16) and the other near the histidine operon (8). Both positions are distinct from a cistron mapped between 16.0 and 16.5 min which includes genes encoding polypeptide components of three other TCA cycle enzymes (34) as well as from the mapped loci for isocitrate dehydrogenase at 25 min (2) and fumarase at 35.5 min (14).

Structural comparisons have led to the suggestion that the cytoplasmic and mitochondrial isozymes of malate dehydrogenase in eucaryotic cells may share a common evolutionary precursor with the *E. coli* enzyme (12). Approximately 20% of the amino acid residues in the eucaryotic isozymes from porcine heart are identical (5), and surprisingly, 69% of the first 36 amino acid residues in *E. coli* and porcine heart mitochondrial malate dehydrogenases are homologous (12). In addition, the sizes of these proteins are similar, with subunit molecular weights of 32,500 for the *E. coli* enzyme (12) and 34,000 and 36,000, respectively, for the pig heart mitochondrial and cytoplasmic isozymes (3). The catalytically active form of all three enzymes is a dimer.

To extend these analyses of the structure, evolution, and expression of malate dehydrogenase, we have used both protein sequence data and genetic information to isolate and subclone the *E. coli* gene encoding this TCA cycle enzyme.

MATERIALS AND METHODS

Strains and media. *E. coli* JA200 (F^+ *trpE5 recA1 leuB6 lacY1*) was used as a host for ColE1 plasmids from the Clarke-Carbon library (7). pUC plasmids (24) were amplified in strain RR101 (F^- *hsdS1 proA2 leu6 ara-14 galK2 lacY1 xyl-5 mtl-1 str-20 thi-1 supE44*) (6). *E. coli* malate dehydrogenase mutants, W945T1-2 (F^- *thr-1 leuB6 trp-59 mdh-2 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL*) (8) and UTH 6276 (F^- *hisG1 argG6 leu-6 metB1 mdh-9*) (16), were kindly provided by B. Bachman and T. Matney, respectively. For selection of ColE1 plasmids, colicin was prepared as described by Schwartz and Helinski (30), and appropriate concentrations were empirically determined by titrations on agar plates containing either LB or minimal medium. Minimal medium was M-9 salts (26) supplemented with (per milliliter) 10 μg of thiamine, 1 mg of Casamino Acids, and 50 μg of each amino acid needed to satisfy auxotrophic requirements. The carbon sources utilized included 0.4% glucose, 0.6% sodium acetate (pH 6.0), and 0.6% L-malate (pH 6.0). Anaerobic cultures were grown in culture tubes filled to the cap with medium and incubated without shaking as described by Courtright and Henning (8).

Malate dehydrogenase assays. Cells were grown to log phase (A_{660}, 0.4 to 0.6) in 10-ml cultures in minimal medium containing various carbon sources. The cultures were chilled on ice for 5 min after the addition of sodium azide to 2 mM and chloramphenicol to 170 μg/ml. Washed cell pellets were suspended in 200 μl of 0.1 M Tris (pH 7.4)–2 mM EDTA–0.2 M dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride–10% glycerol. Cell extracts were prepared by vortexing with glass beads, followed by centrifugation for 10 min in an Eppendorf Microfuge. Nucleic acids were removed by precipitation with 0.2% protamine sulfate and centrifugation. Malate dehydrogenase activity was measured spectrophotometrically after the addition of 10 μl of the crude extract to 1.0 ml of an assay mix containing 50 mM Tris (pH 7.6), 50 mM L-malate (pH 7.0), and 2 mM NAD^+. Protein concen-

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trations were determined by the method of Lowry et al. (22). Specific activity is expressed as micromoles of NADH formed per minute per milligram of protein. Electrophoretic variants of malate dehydrogenase were resolved by electrophoresis of 25-μl samples of crude extracts on 8% polyacrylamide slab gels (10) and visualized by incubation of each gel for 30 min at room temperature in 50 ml of the enzyme assay mix containing 20 mg of Nitro Blue Tetrazolium and 2 mg of phenazine methosulfate.

Oligodeoxynucleotide synthesis. Chemical synthesis of a mixed-sequence oligodeoxynucleotide was done by the phosphotriester solid-phase method developed by Itakura et al. (20). The polystyrene support, mono- and dinucleotide coupling units, and coupling reagent were purchased from Bachemgentec, Inc. Other solvents were obtained from commercial sources and prepared as recommended by the manufacturers (20). After the final coupling reaction, the oligodeoxynucleotide products were removed from the support, dialyzed extensively with 50 mM ammonium bicarbonate, and lyophilized. After electrophoresis of 100-μg samples on 20% polyacrylamide–7.0 M urea gels, the oligodeoxynucleotides were visualized with UV light, and full-length products (14-mers) were excised from the gel and eluted by crushing the gel slices in 10 volumes of TBE buffer (10 mM Tris, 10 mM borate, 0.2 mM EDTA [pH 8.3]). The oligodeoxynucleotide was applied to a Sephadex G-50 column equilibrated with TBE buffer, eluted with TBE buffer containing 1.0 M NaCl, and precipitated at −20°C after the addition of 2 volumes of 95% ethanol.

Nucleic acid isolation and subcloning procedures. Plasmid DNAs were isolated from cleared lysates of amplified cultures by hydroxyapatite chromatography (31). *E. coli* chromosomal DNA was prepared by phenol extraction of lysed cells and RNase treatment as described by Saito and Miura (29). Total cellular RNA was isolated by hot-phenol extraction (26) of cell pellets from 10-ml cultures.

For subcloning, DNA fragments eluted from 1% agarose tube gels were ligated and plasmid vectors digested with appropriate restriction enzymes and treated with calf intestinal phosphatase (Boehringer-Mannheim Biochemicals). DNA ligase was purchased from P-L Biochemicals, Inc. Restriction digests of plasmid DNAs isolated in small-scale preparations (18) were used to screen colonies obtained by transformation with ligation mixes.

Hybridization conditions. Oligodeoxynucleotide probes were prepared by 5′-end labeling with [γ-^{32}P]ATP (>4,000 Ci/mM; ICN Pharmaceuticals, Inc.) and polynucleotide kinase (P-L Biochemicals). For Southern blot hybridizations, DNA fragments were electrophoresed on 0.8% agarose gels and transferred to nitrocellulose filters (33). Filters were prehybridized for 3 h at 22°C in a solution of 5× SSC (1× SSC is 0.15 M sodium chloride–15 mM sodium citrate [pH 7.0]), 0.1 mM ATP, 1.0 mM sodium pyrophosphate, and 0.1% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone and then hybridized for 36 h at 22°C in the same solution containing 20 ng of the ^{32}P-labeled oligodeoxynucleotide. The filters were washed for 5 min at 4°C in 5× SSC containing 0.1 mM ATP and 1.0 mM sodium pyrophosphate, dried, and exposed to Kodak XAR X-ray film at −70°C.

Purified DNA fragments used as hybridization probes were labeled by nick translation with [α-^{32}P] deoxynucleoside triphosphate (28). Southern blot hybridizations with these probes were incubated at 65°C in 3× SSC containing 10 μg of denatured salmon sperm DNA per ml and 0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone. Filters were washed three times in 3× SSC at 65°C. For Northern blots, 20-μg RNA samples were denatured and electrophoresed on formaldehyde gels as described by Hoffman et al. (17). After the samples were transferred to nitrocellulose, hybridizations were conducted for 36 h at 42°C in 5× SSC, 50% formamide, 10 μg of salmon sperm DNA per ml, and 0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone. The filters were washed twice for 5 min per wash at 42°C with 5× SSC, and autoradiography was conducted as described above. Relative levels of hybridization were quantitated by the cutting of bands containing ^{32}P-labeled hybrids from the nitrocellulose filter and scintillation counting.

Restriction mapping and nucleotide sequence analysis. Mapping of restriction endonuclease sites was accomplished by comparing fragments generated in single and double restriction enzyme digests. Partial cleavages of fragments ^{32}P labeled on one end (32) were used to map positions of restriction sites relative to the *Hin*dIII site of pLC32-38H.

For sequence analysis, isolated DNA fragments were treated with phosphatase and 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase. Fragments labeled at one end were generated by cleavage with a second restriction enzyme, subjected to base-specific cleavage reactions (23), and electrophoresed on 8% polyacrylamide–7 M urea sequencing gels.

RESULTS

Malate dehydrogenase expression in transformed cells. Using complementation analysis of *mdh* mutants containing active but electrophoretically variant forms of malate dehydrogenase, Heard et al. (16) mapped the position of the *mdh* locus to 70.6 min on the *E. coli* linkage map. To limit the search for the *mdh* gene, we examined the gene-protein index presented by Neidhardt et al. (27). Only three plasmids in the Clarke-Carbon recombinant library (7) contain chromosomal DNA inserts which have been mapped to this region. Transformants of *E. coli* JA200 carrying each of these plasmids were obtained and tested for malate dehydrogenase activity. Assays of crude extracts prepared from log-phase cultures of each of these transformants indicated that the level of malate dehydrogenase activity in the transformant carrying plasmid pLC32-38 was 10-fold higher (130.5 μmol of NADH formed per mg per min) than that observed for untransformed cells (13.0 μmol/mg per min) or for the other two transformants (11.9 and 8.0 μmol/mg per min for pLC12-8 and pLC21-34, respectively; each value represents an average of two independent determinations; linkage map positions of *E. coli* chromosomal DNA inserts are 70 for pLC12-8 and pLC21-34 and 71 for pLC32-38).

Plasmid pLC32-38 was isolated and used to transform UTH 6276, one of the *mdh* mutants described by Heard et al. (16). Levels of malate dehydrogenase activity measured in extracts from independent transformants were 7- to 10-fold higher than levels measured for the untransformed mutant, and the extracts contained the electrophoretic form of malate dehydrogenase characteristic of the wild-type strain (JA200; data not shown). Similar attempts to complement another *mdh* mutant, W945T1-2, isolated by Courtright and Henning (8), were unsuccessful. This result was not unexpected, since different map positions have been reported for the mutation in this strain which results in a null phenotype for malate dehydrogenase activity (8) and for the chromosomal DNA insert in pLC32-38 (27).

Synthesis of an oligodeoxynucleotide probe for the *mdh*

gene. Fernley et al. (12) reported the amino acid sequence for 36 amino-terminal residues of *E. coli* malate dehydrogenase. To facilitate identification and subcloning of the structural gene presumed to be present on pLC32-38, the nucleotide coding sequence predicted for the pentapeptide containing residues 32 through 36 (LYDIA) was chosen for chemical synthesis of a complementary oligodeoxynucleotide. This sequence is characterized by a high degree of codon bias for leucine and isoleucine (19) and by low codon redundancy for tyrosine and aspartate. These features were considered in limiting the number of potential coding sequences selected for synthesis of the complementary oligodeoxynucleotides shown in Fig. 1. The desired products of this synthesis, 14-mers containing a mixture of 32 nucleotide sequences, were separated from shorter by-products by electrophoresis on a 20% polyacrylamide gel containing 7 M urea and 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase.

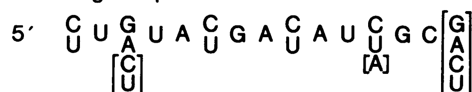
Initial hybridization tests with the labeled probe were conducted with Southern blots of *E. coli* chromosomal DNA fragments produced by digestion with various restriction enzymes, electrophoresed on 0.8% agarose gels, and transferred to nitrocellulose. Optimal conditions for hybridization were empirically determined with a series of identical nitrocellulose filters. The use of [γ-^{32}P]ATP of high specific activity (>4,000 Ci/mmol) in oligodeoxynucleotide kinase reactions was found to be essential for the detection of hybridizing bands. Also, hybridization temperatures of 18 to 24°C followed by brief washes at 4°C in solutions containing 0.1 mM ATP and 1 mM sodium pyrophosphate were required for reproducible hybridization with low background. Under these hybridization conditions, the 5′-end-labeled oligodeoxynucleotide probe was found to hybridize consistently with several restriction fragments in each chromosomal DNA digest (data not shown).

The protocol established for chromosomal DNA digests was followed in a series of dot blot hybridization experiments performed with purified pLC plasmid DNAs. The ^{32}P-labeled *mdh* oligodeoxynucleotide probe was found to hybridize preferentially with pLC32-38 compared with pLC12-8, pLC21-34, and several other colicin plasmids (data not shown). These results, in combination with the observed elevation of malate dehydrogenase activity in pLC32-38 transformants, suggested that the *mdh* structural gene was present on the chromosomal DNA insert of pLC32-38 and could be subcloned with the mixed oligonucleotide probe.

Selected Pentamer:

L Y D I A

Potential coding sequences:



Complementary oligodeoxynucleotides:

FIG. 1. Sequences used in synthesis of an oligodeoxynucleotide probe for the *mdh* gene. The coding sequence for a pentamer (LYDIA = Leu Tyr Asp Ile Ala) within the partial amino acid sequence for *E. coli* malate dehydrogenase reported by Fernley et al. (12) was chosen for synthesis of a complementary oligodeoxynucleotide probe. To limit the number of unique sequences in the product, two possible third-position bases (C and U) in leucine codons, one possible third-position base (A) in isoleucine codons, and the terminal third-position bases in alanine codons were omitted in the synthesis.

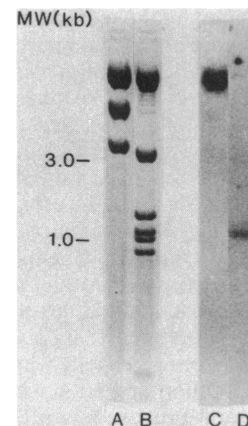


FIG. 2. Localization of pLC32-38 sequences complementary to the *mdh* oligodeoxynucleotide probe. DNA fragments from plasmid pLC32-38 digested with *Hin*dIII (lane A) and *Pvu*II (lane B) were electrophoresed on an 0.8% agarose gel and visualized by ethidium bromide fluorescence. Lanes C and D are autoradiograms of the *Hin*dIII and *Pvu*II cleavage fragments, respectively, after transfer to a nitrocellulose filter and hybridization with the ^{32}P-labeled oligodeoxynucleotide complementary to predicted coding sequences within the *mdh* gene.

Localization and subcloning of the *mdh* gene. Restriction enzyme digestion of plasmid pLC32-38 with *Hin*dIII produces three large DNA fragments (Fig. 2, lane A). Southern blot analysis was used to localize sequences complementary to the *mdh* oligodeoxynucleotide probe to the largest (17-kilobase [kb]) *Hin*dIII fragment (Fig. 2, lane C). Since this fragment also contains the ColE1 plasmid sequences, it was isolated, ligated, and used to retransform JA200. The smaller plasmid, designated pLC32-38H, produced a much higher yield when amplified than did the parental pLC32-38, presumably due to size differences, and was used in subsequent restriction mapping experiments.

Restriction fragments of pLC32-38 generated by digestion with several other endonucleases were analyzed by Southern blot hybridizations with the ^{32}P-labeled *mdh* oligodeoxynucleotide probe. A 1.2-kb *Pvu*II fragment which hybridized with the probe (Fig. 2, lanes B and D) was isolated and cloned into pUC8, producing the plasmid designated pEM1. Partial restriction maps determined for pLC32-38H and pEM1 are shown in Fig. 3. The subcloned *Pvu*II fragment is located 1.8 kb from the unique *Hin*dIII site in pLC32-38H.

Partial nucleotide sequence analysis of the *mdh* gene. In Southern blot analyses, sequences hybridizing with the *mdh* oligodeoxynucleotide probe were further localized to a small *Hae*II fragment (0.2 kb) within the pEM1 subclone. For nucleotide sequence analysis, this *Hae*II fragment was isolated, 5′ end labeled with polynucleotide kinase and [γ-^{32}P]ATP, and digested with *Hin*fI. The *Hae*II-*Hin*fI fragment (Fig. 3) was sequenced by the base-specific cleavage reactions of Maxam and Gilbert (23). The resulting nucleotide sequence and overlapping data obtained from a second fragment (*Pst*I-*Pvu*II) are presented in Fig. 4. This partial nucleotide sequence includes codons for the first 40 amino acids beginning with the amino-terminal methionine. The coding sequence corresponds exactly with the partial amino acid sequence derived through residue 36 for purified *E. coli* malate dehydrogenase (12) and confirms the location of

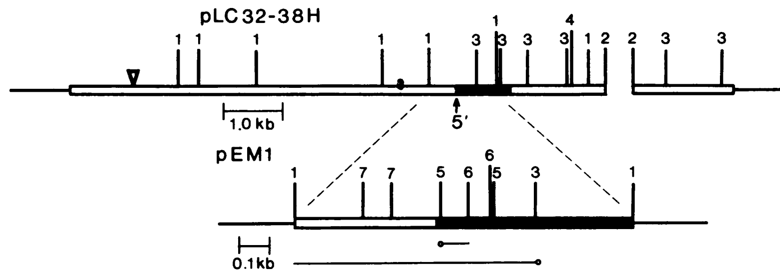


FIG. 3. Partial restriction endonuclease maps for pLC32-38H and pEM1. Vector DNA is indicated by single lines, and *E. coli* chromosomal DNA inserts are indicated by double lines. The 5' end of the *mdh* coding region (shaded) and the direction of transcription (arrow) defined by mRNA size and nucleotide sequence are indicated. Numbers represent restriction endonuclease sites as follows: 1, *Pvu*II; 2, *Hin*dIII; 3, *Pst*I; 4, *Bam*HI; 5, *Hae*II; 6, *Hin*fI; 7, *Hpa*I. The gap at the *Hin*dIII site in pLC32-38H represents the site of ligation of noncontiguous sequences after the removal of two *Hin*dIII fragments from the parental plasmid pLC32-38. Fragments used for nucleotide sequences analysis are indicated below the map for pEM1. The inverted triangle indicates the location of a *Hinc*II recognition site used by Eckhardt as a marker for the *argR* gene (11).

serine residues at positions 26, 28, and 31, which were tentatively assigned in the protein sequence determination. The positions of *Hae*II and *Hin*fI recognition sites establish the orientation of the *mdh* gene on pEM1 and pLC32-38H (Fig. 3). The sequence also includes one of the alternative sequences of codons for the LYDIA pentapeptide used in the synthesis of the *mdh* oligodeoxynucleotide probe (CUGUAUGAUAUCGC−).

Structure of the chromosomal *mdh* gene. To examine the structure of chromosomal *mdh* sequences, the *Hae*II fragment contained within the coding region of pEM1 was isolated and nick translated for use as a hybridization probe. In Southern blots of *E. coli* genomic DNA digested with *Pvu*II, a single 1.2-kb fragment hybridizes with the ^{32}P-labeled *mdh* probe (Fig. 5A). This fragment is presumably the same as that which was subcloned from pLC32-38. Similarly, other restriction enzymes generated single hybridizing fragments (data not shown), suggesting there is a single structural gene for *mdh* in the *E. coli* genome. In contrast with the unique genomic DNA fragments which hybridize with the ^{32}P-labeled *Hae*II fragment, as mentioned above, several genomic fragments from similar restriction digests were found to hybridize with the ^{32}P-labeled synthetic oligodeoxynucleotide. This hybridization probably reflects homology between several genomic sequences and various sequences contained within the mixed oligodeoxynucleotide.

Expression of the *mdh* gene. To examine expression of the *mdh* gene, malate dehydrogenase activity and mRNA levels were analyzed in cells grown under various cultivation conditions as described in Materials and Methods. For these experiments, a crude extract for enzyme assays was prepared from cells in one 10-ml sample, and total cellular RNA was isolated from cells in another 10-ml sample of each culture. Relative levels of malate dehydrogenase activity measured in crude extracts are shown in Table 1. The lowest specific activity was observed in extracts from JA200 cells cultivated under anaerobic conditions. Under aerobic conditions, the specific activity of malate dehydrogenase was fourfold higher with acetate and twofold higher with malate than with glucose as a carbon source. The specific activities measured in extracts of JA200 cells transformed with pLC32-38 were three- to fourfold higher than levels for untransformed cells cultivated under similar conditions with the exception of a 10-fold increase with aerobic growth on glucose. Thus, comparable levels of malate dehydrogenase activity are observed for transformants grown aerobically on acetate or glucose.

To determine whether differences in specific activities observed for JA200 cells cultivated under various conditions could be correlated with different relative levels of malate dehydrogenase mRNA, RNA samples were examined by Northern blot analysis as described in Materials and Methods by using as a hybridization probe the ^{32}P-labeled *Hae*II fragment isolated from the *mdh* coding region on pEM1. The major RNA species from untransformed cells which hybridized with the *mdh* probe migrates under these electrophoretic conditions slightly ahead of 16S rRNA (1,542 base pairs; Fig. 5B). Since the polypeptide chain contains 314 amino acids as judged by amino acid composition (12), this transcript is in the appropriate size range for a monocistronic mRNA for malate dehydrogenase. Relative levels of the

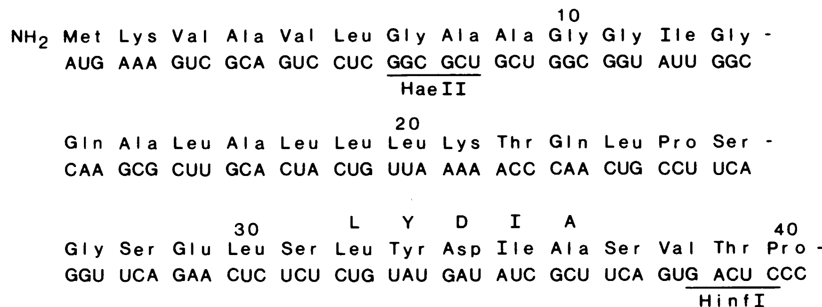


FIG. 4. Partial nucleotide sequence of *mdh*. The partial amino acid sequence (through residue 36) previously determined for *E. coli* malate dehydrogenase (12) is compared with the 5' nucleotide coding sequence obtained in this study.

transcript in each lane were quantitated as described in Materials and Methods. The hybridizing transcript appears to be threefold more abundant in RNA from acetate- (Fig. 5B, lane b) as compared with glucose- or malate-grown cells (lanes a and c, respectively). In contrast, a transcript complementary to the *mdh* probe is barely detectable in RNA from an anaerobic culture of JA200 (lane d). In addition, hybridization to a transcript of the same size in RNA samples prepared from JA200 cells transformed with pLC32-38 is increased three- to fivefold over levels for untransformed cells grown under similar cultivation conditions (data not shown). Thus, the relative levels of malate dehydrogenase mRNA correspond well with measured levels of enzyme activity.

TABLE 1. Malate dehydrogenase activity in JA200 cells and pLC32-38 transformants under various cultivation conditions

Plasmid	Cultivation condition		Activity (U/mg)[a]
	Carbon source	Aeration	
None	Glucose	Aerobic	13.0
	Acetate	Aerobic	54.6
	Malate	Aerobic	24.2
	Glucose	Anaerobic	3.2
pLC32-38	Glucose	Aerobic	130.5
	Acetate	Aerobic	156.0
	Malate	Aerobic	78.3
	Glucose	Anaerobic	12.6

[a] Units are expressed as micromoles of NADH formed per minute. Each value represents an average of two independent determinations.

DISCUSSION

Using a synthetic oligodeoxynucleotide hybridization probe in combination with enzymatic assays of transformed cells, we have identified and subcloned the structural gene for malate dehydrogenase from *E. coli*. Identification of the *mdh* gene on the chromosomal DNA insert of plasmid pLC32-38 from the Clarke-Carbon library (7) confirms the map position deduced from complementation studies by Heard et al. (16), which is distinct from a cistron encoding other TCA cycle enzymes, including citrate synthase and components of succinate and α-ketoglutarate dehydrogenases (34). Few other genetic loci have been formally identified in regions near the *mdh* locus (27). However, the arginine repressor gene (*argR*) has also been isolated from pLC32-38 (11). By comparing restriction maps, we have located the *Hinc*II restriction site used in that report as a marker for the *argR* gene at a position approximately 5 kb from the 5′ end of the *mdh* gene (Fig. 3).

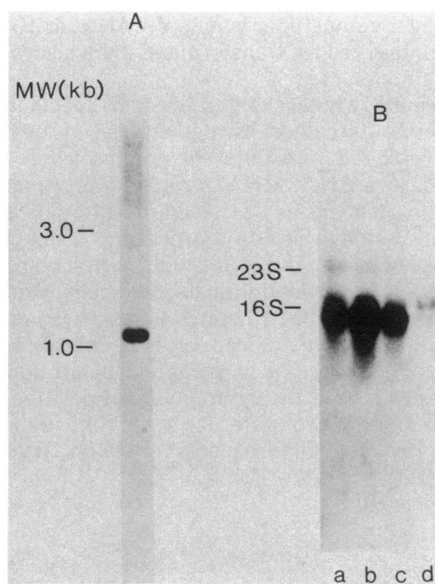


FIG. 5. Hybridization of *E. coli* DNA and RNA with a ^{32}P-labeled DNA fragment isolated from the *mdh* gene. The 0.2-kb *Hae*II fragment within the coding region of *mdh* of pEM1 (Fig. 3) was isolated, ^{32}P labeled by nick translation, and hybridized with 5 μg of *E. coli* DNA that had been digested with *Pvu*II, electrophoresed on an 0.8% agarose gel, and transferred to nitrocellulose (A). The same ^{32}P-labeled *mdh* fragment was hybridized with 20-μg RNA samples electrophoresed on formaldehyde-agarose gels and transferred to nitrocellulose (B). RNA samples were isolated from JA200 cells grown under aerobic conditions with glucose (lane a), acetate (lane b), or malate (lane c) and under anaerobic conditions with glucose (lane d) as the carbon source.

In an earlier analysis of *mdh* mutants, Courtright and Henning (8) concluded that the *mdh* gene was located near the histidine operon. However, we have been unable to complement the phenotypic defect of their mutant by transformation with plasmids carrying the *mdh* structural gene. Thus, this strain may harbor a mutation in a locus which regulates malate dehydrogenase expression or activity.

Analyses of expression of the *mdh* gene suggest that mRNA and active enzyme are most abundant in cells grown with acetate as a carbon source. The lower levels of expression observed with malate may reflect a need for increased production of oxaloacetate for optimal function of the glyoxylate pathway during growth on acetate. The threefold reduction in *mdh* expression observed in aerobic glucose-grown cells probably reflects catabolite repression. We have observed that this repression is more dramatic in rich medium (unpublished observations), which presumably alleviates requirements for biosynthetic functions of TCA cycle enzymes (13). The extent of repression during aerobic growth on glucose is apparently reduced in cells transformed with plasmid pLC32-38 carrying the *mdh* structural gene (Table 1). In contrast, under anaerobic conditions, malate dehydrogenase activity was dramatically reduced to similar relative levels in both untransformed and transformed cells. This result suggests a saturation of repression by the plasmid under aerobic but not anaerobic conditions.

The detection of very low levels of malate dehydrogenase mRNA and enzyme activity in anaerobic cultures complements the observation that *mdh* mutants can grow anaerobically with glucose as a carbon source (8). These results suggest a modification of the proposal by Amarasingham and Davis (1) that the TCA cycle becomes a branched pathway during anaerobiosis to provide for the biosynthesis of α-ketoglutarate (through sequential reactions catalyzed by citrate synthase, aconitase, and isocitrate dehydrogenase) and of succinate (through sequential reactions catalyzed by malate dehydrogenase, fumarase, and succinate dehydrogenase). Since malate dehydrogenase and fumarase (8) are repressed under anaerobic conditions, it is likely that succinate is produced by another mechanism. Courtright and Henning (8) have suggested that succinate may be derived from aspartate through reactions catalyzed by aspartase and fumarate reductase. The reaction catalyzed by isocitrate lyase can also generate succinate during anaerobic growth (9). Thus, if α-ketoglutarate is synthesized as proposed (1),

anaerobiosis may be one condition which results in differential expression of TCA cycle genes.

A partial nucleotide sequence has confirmed the identity and physical orientation of the *E. coli mdh* gene. This sequence analysis will be completed to determine the extent of homology between procaryotic and eucaryotic mitochondrial malate dehydrogenases. The homology noted in a comparison of amino-terminal residues (12) established a similarity within the nucleotide-binding domains of these proteins. It will be of interest to determine whether residues implicated in the active site and in subunit-subunit interactions for porcine heart mitochondrial malate dehydrogenase (4) have also been conserved. In addition, analyses of sequences flanking the coding region may extend understanding of the regulation of expression of this TCA cycle enzyme. Of particular interest will be a comparison of *mdh* sequences with putative regulatory sequences reported for the *E. coli* gene encoding fumarase (25) to determine if features of coordinate regulation (i.e., repression during anaerobiosis) can be defined at a structural level.

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