

Functions of the Membrane-Associated and Cytoplasmic Malate Dehydrogenases in the Citric Acid Cycle of *Escherichia coli*

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Received 17 May 2000/Accepted 21 September 2000

Oxidation of malate to oxaloacetate in *Escherichia coli* can be catalyzed by two enzymes: the well-known NAD-dependent malate dehydrogenase (MDH; EC 1.1.1.37) and the membrane-associated malate:quinone-oxidoreductase (MQO; EC 1.1.99.16), encoded by the gene *mgo* (previously called *yojH*). Expression of the *mgo* gene and, consequently, MQO activity are regulated by carbon and energy source for growth. In batch cultures, MQO activity was highest during exponential growth and decreased sharply after onset of the stationary phase. Experiments with the β -galactosidase reporter fused to the promoter of the *mgo* gene indicate that its transcription is regulated by the ArcA-ArcB two-component system. In contrast to earlier reports, MDH did not repress *mgo* expression. On the contrary, MQO and MDH are active at the same time in *E. coli*. For *Corynebacterium glutamicum*, it was found that MQO is the principal enzyme catalyzing the oxidation of malate to oxaloacetate. These observations justified a reinvestigation of the roles of MDH and MQO in the citric acid cycle of *E. coli*. In this organism, a defined deletion of the *mdh* gene led to severely decreased rates of growth on several substrates. Deletion of the *mgo* gene did not produce a distinguishable effect on the growth rate, nor did it affect the fitness of the organism in competition with the wild type. To investigate whether in an *mgo* mutant the conversion of malate to oxaloacetate could have been taken over by a bypass route via malic enzyme, phosphoenolpyruvate synthase, and phosphoenolpyruvate carboxylase, deletion mutants of the malic enzyme genes *sfcA* and *b2463* (coding for EC 1.1.1.38 and EC 1.1.1.40, respectively) and of the phosphoenolpyruvate synthase (EC 2.7.9.2) gene *pps* were created. They were introduced separately or together with the deletion of *mgo*. These studies did not reveal a significant role for MQO in malate oxidation in wild-type *E. coli*. However, comparing growth of the *mdh* single mutant to that of the double mutant containing *mdh* and *mgo* deletions did indicate that MQO partly takes over the function of MDH in an *mdh* mutant.

In *Escherichia coli*, several enzymes or pathways are able to convert malate to oxaloacetate. The NAD-dependent (cytoplasmic) malate dehydrogenase (MDH; EC 1.1.1.37) has always been considered to be the principal malate-oxidizing enzyme in the citric acid cycle (tricarboxylic acid [TCA] cycle) of this organism (9). Recently, a malate:quinone-oxidoreductase (MQO; EC 1.1.99.16) which is an essential enzyme in the TCA cycle of *Corynebacterium glutamicum* (reference 23 and accompanying paper) was described. MQO is a flavin adenine dinucleotide (FAD)- and lipid-dependent peripheral membrane protein catalyzing the oxidation of L-malate to oxaloacetate. The electrons are donated to the electron transfer chain at the level of quinones. This reaction is essentially irreversible (17). In *C. glutamicum*, MQO and the cytoplasmic NAD-dependent malate dehydrogenase, which is capable of reversible oxidation of malate to oxaloacetate, are active at the same time. Due to the different natures of their electron acceptors, the Gibbs standard free energies for the reactions catalyzed by MQO and MDH differ radically. For the MDH-dependent reaction, the standard free energy is equal to $+28.6 \text{ kJ} \cdot \text{mol}^{-1}$, and for the MQO-dependent reaction, it is -55 or $-18.9 \text{ kJ} \cdot \text{mol}^{-1}$, depending on whether the acceptor is ubiquinone or menaquinone, respectively (23). These figures suggest that, when both enzymes are active, under standard-state conditions, MQO should catalyze the oxidation of malate whereas MDH should catalyze the reduction of oxaloacetate. In *C. glutamicum*, it was, indeed, clearly demonstrated that MQO is the principal

enzyme responsible for the oxidation of malate to oxaloacetate, whereas there were indications that MDH catalyzes oxaloacetate reduction (accompanying paper). MDH could be forced to function in the direction of malate oxidation only when the *mgo* gene was deleted and the NAD concentration in the cell was increased by addition of nicotinamide to the growth medium.

In *E. coli*, an MQO-like enzyme activity (at the time usually referred to as malate oxidase) was described before. This activity was thought to be induced to significant levels only in certain mutants lacking MDH activity (12). Because an MDH-negative mutant which still contained inactive MDH protein exhibited very high MQO activity, it was surmised that the expression of the gene encoding MQO was regulated by substrates or products of the MDH reaction (25). MQO was partially purified from an MDH-negative *E. coli* strain which overexpressed this enzyme (25). Properties of the partially purified protein were similar to those found for the purified protein of *C. glutamicum* (23). Initial studies by us, using a more sensitive MQO assay and optimized assay conditions (unpublished results), indicated that in contrast with previous observations MDH and MQO were active at the same time in *E. coli*. Because of this observation, and regarding the findings in *C. glutamicum*, a reinvestigation of the role of MDH and more detailed studies of the role of MQO in *E. coli* were deemed necessary.

The open reading frame (ORF) *yoyH* (EMBL accession no. AE000310) found on the genome of *E. coli* has a high similarity (43.9% identical residues) to the *mgo* gene of *C. glutamicum* (23). This ORF (here called *mgo*) was used to overexpress MQO activity in *E. coli*. Furthermore, by using a construct of the *mgo* promoter region fused with the reporter gene *lacZ*,

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TABLE 1. Oligonucleotides used for PCRs

Designation	Sequence	bp on <i>E. coli</i> chromosome (2) ^a
Y_01	5'-GCTGGATGAATGGGCGGCGG-3'	c2305202-2305221
Y_04	5'-CGCGGATCCCCGGTTTCAACGATGATG-3'	2302220-2302240
YojH_01	5'-GGATCCGTTTCATGCCGCGCAAATC-3'	c2304816-2304829
YojH_02	5'-GTTACGCCGCATCCAACATC-3'	2303020-2303039
F_01	5'-GGGGCCGGAGGGTAAACC-3'	c2305381-2305398
F_02	5'-GAATCCGTAATCATGGTCATCTTGTAAATGCCTTAC-3'	365511-365530 and 2304772-2304791
F_03	5'-GTAAGGCATTAACAAGATGACCATGATTACGGATTC-3'	c365511-365530 and c2304772- 2304791
F_04	5'-GCGGAATTCTTAAACGCCATCAAAAATAATTTCG-3'	365090-365110
Komdh_01	5'-CGCGGATCCGCGAGACTTTAGACT-3'	c3382466-3382484
Komdh_02	5'-CGCGAATCCCAACAGGTGGTGAC-3'	3382097-3382114
Komdh_03	5'-CGCGAATCCCTGAATGGTGACCTGCAG-3'	c3380865-3380886
Komdh_04	5'-CGCGGATCCGGTGTAATCATGGC-3'	3380073-3380091
Mao1_01	5'-GCATGGATCCAGTATCAGG-3'	c31653499-31653517
Mao1_02	5'-GCGGATCCGGCAGTACCATAACCTTC-3'	1552163-1552182
Mao2_01	5'-CCGGGATCCTCAACGGCTTGCGC-3'	c2575862-2575879
Mao2_02	5'-CAGGATCCATGTTCCGGCATCACC-3'	2574297-2574314
Pps_01	5'-CGCGGATCCCGAGTGGATTAC-3'	c1784850-1784867
Pps_02	5'-CGCGGATCCCGACATATTTGCC-3'	1782889-1782906

^a c, complement.

the regulation of expression was examined. To investigate the importance of MQO and MDH for the physiology of *E. coli*, and to investigate the supposed regulation of *mgo* expression by MDH, deletions of *mgo* and *mdh* were constructed on the chromosome. To assess the roles of MDH and MQO in malate oxidation, site-directed deletions of the genes coding for the malic enzymes and for phosphoenolpyruvate (PEP) synthase were constructed to block alternative metabolic routes leading from malate to oxaloacetate.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5 α (*supE44* Δ *lacU169* Φ 80*lacZ* Δ M15 *hsdR17* *recA1* *endA1* *gvrA96* *thi-1* *relA1*) (31) was used for standard genetic experiments. Measurement of MQO activity and gene replacements were carried out in *E. coli* K-12 strains DSM 5698 (ATCC 25404) (F⁻ λ ⁻) and MC4100 [F⁻ λ ⁻ *araD139* Δ (*argF-lac*) *U169* *rpsL150* *flb5301* *deoC1* *ptsF25* *rbsR*] (4, 30). *E. coli* PC2 (parent, MC4100; Δ *frr2*) and PC35 (parent, MC4100; Δ *arcA* Kan^r) were a generous gift from R. P. Gunsalus (7).

Cells were routinely grown on Luria broth (LB) (31) or on Neidhardt minimal medium (NMM) (26) containing carbon source as indicated. When required, the media were supplemented with 100 μ g of carbenicillin per ml or 50 μ g of kanamycin per ml. Controlled batch fermentations were carried out in a 1.5-liter vessel. The culture was maintained at 37°C, and oxygen was maintained at 5.5 mg of O₂ (\pm 10%) liter⁻¹ by regulating the sparge flow.

Construction of pECmgo. Oligonucleotides used are listed in Table 1. The *mgo* ORF (EMBL accession no. AE000310) was amplified by PCR from the *E. coli* MC4100 genome using YojH_01 as forward primer and YojH_02 as reverse primer. PCR conditions were a standard 30 cycles consisting of a temperature profile of 30 s at 94°C, 30 s at 60°C, and 2 min at 72°C. The YojH_01 primer is complementary to a region -101 bp in front of the +1 ATG start codon of the *mgo* ORF, and YojH_02 is located 92 bp following the TAA stop codon. The resulting PCR fragment was cloned in the vector pET324 (36), resulting in pECmgo for expression in *E. coli*. For expression in *C. glutamicum*, the fragment was cloned in pJCBW1, a derivative of pJCI (8, 20) containing the *lacZ* complementation region and multiple cloning site from pSK+ (Stratagene), using *EcoRV*.

Construction of Φ (*mgo-lacZ*). Oligonucleotides used are listed in Table 1. A construct was made in which the *lacZ* gene was fused to the promoter region of *mgo*. Since the *mgo* ORF has at least four possible start sites (ATG), and since it is unclear which one is used by the organism, the most probable start had to be determined by an educated guess. To this end, the hypothetical protein sequence of the *mgo* ORF was compared to that of MQO from *C. glutamicum* (23). The amino-terminal amino acid sequence of the purified MQO protein of *C. glutamicum* was SDS-PAGE (D. Molenaar, unpublished results). This sequence can be recognized in the translated DNA sequence of the *C. glutamicum* *mgo* region and is preceded there by a methionine (see Fig. 5 in reference 23), which is appar-

ently posttranslationally removed. The MQO protein from *C. glutamicum* therefore starts with the methionine immediately in front of a putative FAD binding site referred to in reference 23. The analogous methionine (ATG codon) in the *mgo* ORF, immediately in front of its putative FAD binding site, was defined as the most probable start of MQO of *E. coli*, and the corresponding hypothetical protein starts with the amino acid sequence MAAKA (see Fig. 5 in reference 23). The fusion of the *mgo* promoter region with the *lacZ* gene was constructed in such a way that the *lacZ* ORF starts with the ATG codon of the *mgo* ORF corresponding to this most probable start. A 681-bp region preceding this ATG codon of the *mgo* ORF was isolated using primer F_01 as forward primer and primer F_02 as reverse primer in reaction A. Since the most probable ATG is also the one furthest downstream in the sequence of possible ATG starts, all regulatory elements of the *mgo* promoter should be present in the construct, even if this ATG is not the true start codon. The *lacZ* ORF was isolated using the primer F_03 as forward primer and the primer F_04 as reverse primer in reaction B. The 3' region of primer F_02 and the 5' region of primer F_03 are complementary. In a crossover PCR, the products of reactions A and B are combined with the primers F_01 and F_04. The resulting PCR product is the desired fusion between the promoter region of the *mgo* ORF and the *lacZ* ORF starting with the most probable ATG start codon. The PCR product was digested with *Hind*III and *Eco*RI and inserted into the vector pBR322 (3), resulting in the plasmid pML2.

Construction of *E. coli* *mdh*, *mgo*, *sfcA*, *b2463*, and *pps* deletion strains. Oligonucleotides used are listed in Table 1. PCR conditions were chosen as described above. Chromosomal gene disruptions were carried out in *E. coli* MC4100 according to the method of Link et al. (19).

The region in front of the *mdh* ORF (EMBL accession no. M24777) was amplified by PCR using the oligonucleotides Komdh_01 and Komdh_02 (reaction 1). The region following the *mdh* ORF was amplified using oligonucleotides Komdh_03 and Komdh_04 (reaction 2). The 3' end of Komdh_02 and the 5' start of Komdh_03 are complementary. Using crossover PCR with the products of reactions 1 and 2 and primers Komdh_01 and Komdh_04, a 1,100-bp PCR fragment was created, joining the regions on the *E. coli* genome in front of and behind the *mdh* ORF. The fragment was inserted into the *Bam*HI site of the vector pKO3 (19). The resulting plasmid pMDH was used for gene disruption on the chromosome according to the protocol described by Link et al. (19).

For the disruption of the *mgo* ORF, a 3,002-bp fragment containing the *mgo* ORF was amplified using the oligonucleotides Y_01 and Y_04. Both oligonucleotides introduce *Bam*HI sites. The amplified fragment was digested with *Bam*HI and inserted into the *Bam*HI site of pKO3. The resulting plasmid was digested with *Mlu*I to remove a 416-bp fragment from the *mgo* ORF, thereby disrupting the ORF. The disrupted *mgo* ORF in plasmid pKO3 was used for gene disruption on the chromosome.

The ORF coding for the NAD-dependent malic enzyme A (EMBL accession no. AE000245) was amplified from the *E. coli* chromosome using the oligonucleotides Mao1_01 and Mao1_02. The Mao1_01 oligonucleotide contains a *Bam*HI restriction site, and the oligonucleotide Mao1_02 introduces a *Bam*HI restriction site following the *sfcA* ORF. The resulting PCR fragment was digested with *Bam*HI and ligated into the pKO3 vector opened with *Bam*HI. The

resulting plasmid was digested using the restriction enzyme *PvuI*, thereby deleting a 717-bp fragment from the *sfcA* ORF. The disrupted *sfcA* ORF in pK03 was used for gene disruption. The NADP-dependent malic enzyme (EC 1.1.1.40) is not annotated on the *E. coli* chromosome. The enzyme encoded by *b2463* (EMBL accession no. AE000333) was suggested elsewhere to be an NADP-linked malic enzyme (21), but no experimental data were provided. Using the oligonucleotides Mao2_01 and Mao2_02, the *b2463* ORF was amplified from the *E. coli* chromosome. Both oligonucleotides introduce a *Bam*HI site flanking the PCR fragment. These *Bam*HI sites were used to clone the fragment into the pK03 vector. The resulting plasmid was digested with *NdeI*, thereby removing a 1,078-bp fragment from the ORF. The disrupted ORF in pK03 was used for gene disruption on the chromosome.

The ORF *pps* (EMBL accession no. M69116) coding for the PEP synthase was amplified from the *E. coli* chromosome using the oligonucleotides Pps_01 and Pps_02. Both oligonucleotides introduce a *Bam*HI restriction site in front of and following the *pps* ORF. The resulting PCR fragment was digested with *Bam*HI and the restriction enzymes *HpaI* and *DraI* to delete a 1,038-bp fragment from the *pps* ORF. The *Bam*HI-digested fragments were ligated into the pK03 vector opened with *Bam*HI. The disrupted *pps* ORF in pK03 was used for gene disruption on the chromosome.

Preparation of membrane fragments. A sample of 5×10^{11} cells (calculated by assuming that an optical density at 600 nm equal to 1 corresponds to approximately 5×10^8 cells ml^{-1}) was harvested and washed twice with 25 ml of ice-cold buffer A consisting of 50 mM HEPES, 10 mM K-acetate, 10 mM CaCl_2 , and 5 mM MgCl_2 titrated with NaOH to pH 7.5. The washed cells were resuspended in 10 ml of buffer A and passed once through a French pressure cell at 10,000 lb/in² (69 MPa). Cell debris was removed by centrifuging it for 10 min at $10,000 \times g$. The supernatant was centrifuged for 30 min at $75,000 \times g$, and membranes were resuspended in buffer A to 10 to 15 mg of protein per ml.

Enzyme assays. Malate dehydrogenase activity was determined in cells grown overnight in LB. The cells were washed twice in 150 mM Tris–15 mM EDTA, titrated to pH 7.4 with acetic acid, and passed once through a French pressure cell at 10,000 lb/in² (69 MPa). After a low-speed spin at $2,500 \times g$, the cell extract was used for the assay. Malate dehydrogenase activity was assayed at pH 9.0 in the direction of malate oxidation as described in reference 32.

NADP-dependent malic enzyme activity was measured in cell extract using the method described by Diesterhaft and Freese (11).

MQO activity in membrane fragments was measured using 2,6-dichlorophenolindophenol (DCPIP) as an electron acceptor as described before (23). The reaction mixture additionally contained 30 μM ubiquinone 1.

β -Galactosidase activity was measured according to the method of Sambrook et al. (31).

Competition experiments with wild type and Δmgo . The strain MC4100 and its derivative Δmgo were precultured overnight on LB, and the optical densities at 600 nm of these cultures were measured. The cultures were then mixed to obtain a 1:1 proportion of MC4100 and Δmgo cells. Of this mixture, 2 μl was used to inoculate 2 ml of NMM (26) with different carbon and energy sources. For each carbon and energy source, five cultures were inoculated and maintained in parallel throughout the experiment. The cultures were incubated in a shaker at 37°C for 24 h, and approximately 2 μl of each culture was transferred to 2 ml of fresh NMM. The optical density at 600 nm was measured, and the rest of the culture volume was kept frozen at -20°C . The transfer to fresh medium was repeated seven times. The number of generations after the last cultures had reached stationary phase was approximately 50 to 60. PCR was carried out as described above on 1 μl of a 50-fold-diluted sample of the culture to estimate the proportions of MC4100 and *mgo* using oligonucleotides Yojh_01 and Yojh_02. The linear quantitative character of the PCR method was confirmed by testing with defined mixtures of these strains.

Estimation of growth rates. Two growth curves were recorded by monitoring the optical density at 600 nm of cultures which had been inoculated with overnight cultures on the same medium from two different colonies. The specific growth rates were estimated by simultaneous least-squares fitting of both growth curves to exponential growth equations.

Protein determination. Protein was determined with bicinchoninic acid in the presence of 0.5% (wt/vol) sodium dodecyl sulfate, according to a protocol adapted from reference 34.

RESULTS

The gene *mgo*, formerly called *yojH*, encodes an MQO in *E. coli*. The high amino acid sequence similarity of MQO from *C. glutamicum* and the hypothetical protein encoded by the gene *mgo* (previously called *yojH*) of *E. coli* suggested that this gene encodes an MQO in *E. coli* (23). To test this possibility, the *mgo* gene was amplified by PCR. The PCR product containing the *mgo* ORF was inserted in a high-copy-number vector under the control of the *trc* (*lac*) promoter, resulting in plasmid pECmgo. This plasmid was introduced in *E. coli* DSM 5698 (wild-type K-12 strain). The resulting strain, when grown on

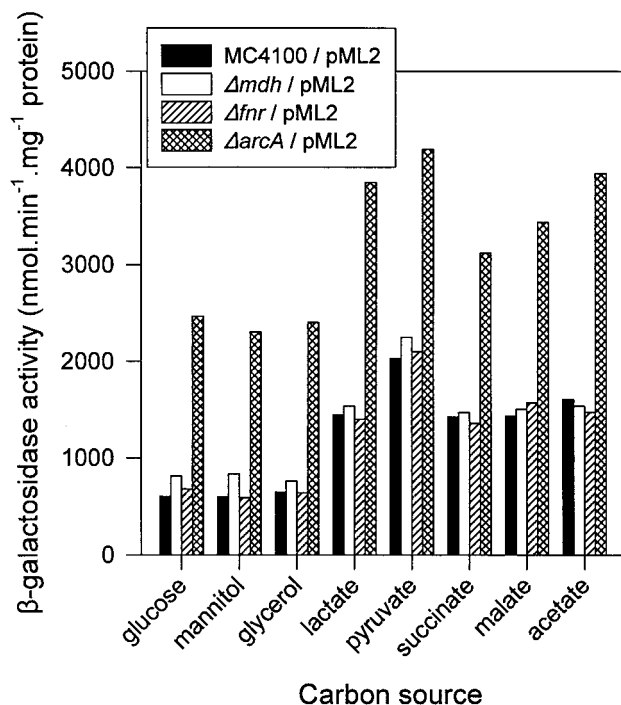


FIG. 1. Expression of $\Phi(mgo-lacZ)$ from plasmid pML2 in *E. coli* MC4100 and in strains lacking *ArcA*, *Fnr*, or *MDH*. Cells were grown in NMM containing the indicated carbon source (0.5% [wt/vol]) to an optical density at 600 nm of 0.4. Hydrolysis of *ortho*-nitrophenyl- β -D-galactopyranoside (ONPG) was used as a measure of β -galactosidase activity (31).

LB, had an MQO activity of 32 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein. The detection of activity in *E. coli* membranes with DCPIP as electron acceptor was absolutely dependent on the addition of a quinone and varied with the particular quinone added. The highest activity is found in the presence of ubiquinone 1 (results not shown). Addition of the inducer isopropyl- β -D-thiogalactopyranoside (IPTG) increased the activity to 176 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein. Using a *C. glutamicum*-*E. coli* shuttle plasmid, pJCBW1, the *mgo* gene could also be expressed in the *C. glutamicum* DM22 mutant which lacks MQO activity (23). The addition of IPTG to these cells gave rise to MQO activity, albeit with lower specific activities than those found in membranes from *C. glutamicum* strain ATCC 13032 (wild type).

Effects of carbon substrates on $\Phi(mgo-lacZ)$ expression. To determine the effect of the carbon source on *mgo* gene expression, an *mgo-lacZ* fusion $\Phi(mgo-lacZ)$ was constructed on a plasmid (pML2). Strain MC4100/pML2, lacking the *lacZ* gene on the chromosome, was grown in NMM on different carbon sources to mid-logarithmic phase, and β -galactosidase levels were determined. Expression was low on NMM with glucose and high when nonfermentable substrates were used as the carbon source (Fig. 1). Expression of *mgo* appears to be under catabolite control, similar to the regulation of *mdh* and the succinate dehydrogenase gene (*sdh*) (28, 29). Expression of $\Phi(mgo-lacZ)$ in a $\Delta arcA$ strain (PC35 [7]) was increased approximately two- to fourfold (Fig. 1). This shows that *mgo*, like many other genes encoding enzymes from the electron transfer chain, is regulated by the *ArcA*-*ArcB* two-component system. Deletion of *fir* had no effect on expression of $\Phi(mgo-lacZ)$, neither under aerobic conditions (Fig. 1) nor under anaerobic growth on glucose. The β -galactosidase levels were approxi-

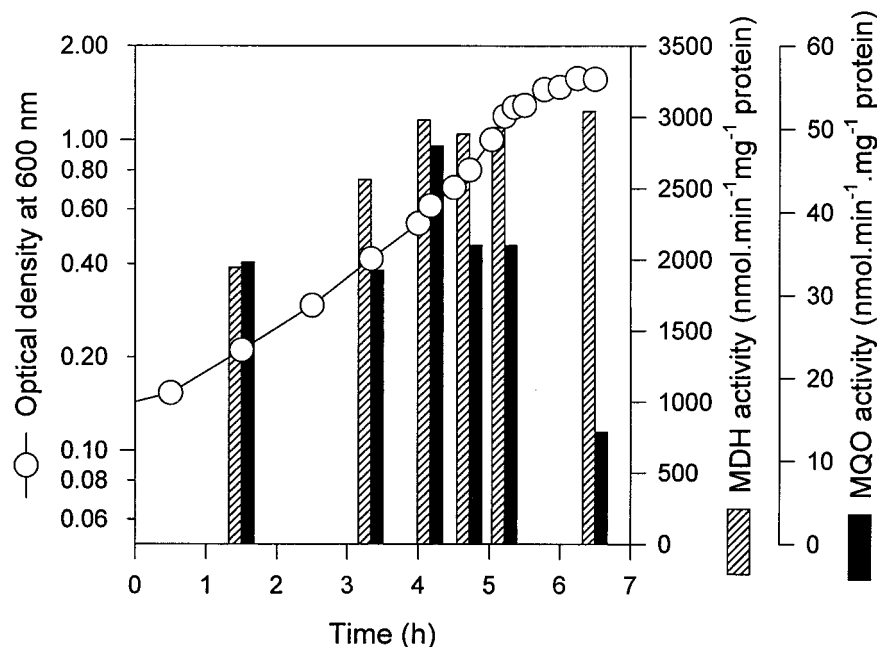


FIG. 2. Effect of the growth phase on MQO activity. *E. coli* MC4100 was grown in NMM using 0.5% (wt/vol) pyruvic acid as carbon source in a controlled batch under constant oxygen pressure. Membrane vesicles and cleared extracts were prepared from cells harvested at indicated positions. MQO and MDH were measured as described in the text.

mately 10-fold lower under anaerobic conditions (data not shown).

Earlier investigations suggested that MQO in *E. coli* was present in only (some) mutants lacking the cytoplasmic malate dehydrogenase (12, 25). These authors interpreted the results as indicating that there existed a repressing effect of MDH on MQO expression, which was dependent on the presence of active MDH. The observed increase of MQO activity in MDH mutants was by at least a factor of 10 to 50 and in one case of 200 to 300 (25). A basis for this regulation was not found, and in this study it is shown that MQO and MDH are active at the same time in *E. coli* (Fig. 2). The mutants used in the earlier studies were generated by random mutation using nitrosoguanidine and are therefore ill defined. The defined *mdh* knockout strain described in this study allowed a clear-cut evaluation of the effect of the absence of MDH activity on MQO expression. Expression of $\Phi(mqo-lacZ)$ in the Δmdh strain resulted in only slightly increased β -galactosidase levels (Fig. 1). MQO activity was increased by at most a factor of 2 in the *mdh* strain in the exponential phase during growth on NMM with glucose. Finally, deletion of the *mqo* gene also had no effect on $\Phi(mqo-lacZ)$ expression (data not shown), ruling out the idea that MQO regulates its own synthesis.

Regulation of MQO activity in *E. coli* by ArcA, carbon source, and growth phase. Since expression of MQO in *E. coli* appears to be highly regulated by carbon source, membrane vesicles were prepared from cells grown on different carbon sources. The cells were harvested from cultures in the exponential growth phase, in the end-exponential phase, and in the stationary phase and from overnight cultures. High MQO activity could be measured in vesicles prepared from exponentially growing cells using pyruvate and acetate as carbon source (Table 2). Cells grown on glucose contained low MQO activities. The difference between MQO activity of cells grown on glucose and activity of cells grown on pyruvate is approximately fourfold and is in accordance with the results obtained for the expression of $\Phi(mqo-lacZ)$ (Fig. 1). In cells grown on glucose, the MQO activity was fourfold higher in the $\Delta arcA$ strain, in accordance with the $\Phi(mqo-lacZ)$ promoter studies. Also, succinate dehydrogenase and MDH were derepressed in the $\Delta arcA$ strain (results not shown and references 28 and 29). In cells growing on LB supplemented with 0.1% glucose, MQO activity can also be measured, in contrast to earlier observations (25).

The growth-phase-dependent MQO activity (Table 2) could be a result of oxygen limitation which occurs at the high cell

TABLE 2. MQO activity in *E. coli* vesicles prepared from cells grown on different media and different carbon sources and harvested at different phases of the growth curve

Growth phase	MQO activity (nmol · min ⁻¹ · mg of protein ⁻¹) on medium:			
	NMM + glucose	NMM + pyruvate	NMM + acetate	LB + glucose
Exponential	12.0	41.3	47	16.1
End exponential	13.7	23.4	4.9	ND ^a
Stationary	9.2	5.7	4.9	5.1
Overnight	4.1	1.9	0.0	0.0

^a ND, not determined.

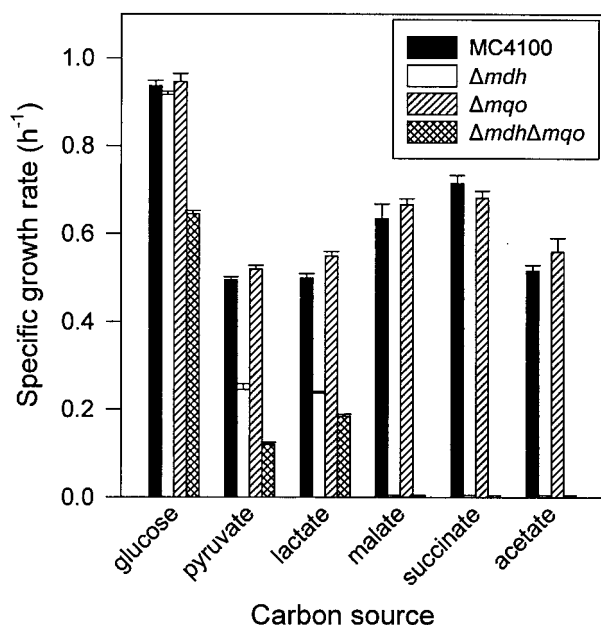


FIG. 3. Effect of *mgo* and *mdh* deletions on the specific growth rate of *E. coli* on various carbon sources. Cells were grown on NMM containing carbon sources (0.5% [wt/vol]) as indicated. Error bars represent estimated standard deviations.

densities during stationary phase and in overnight cultures. In order to avoid oxygen limitations, MQO activity was measured in vesicles prepared from cells grown on pyruvate in a batch culture under controlled high oxygen pressure. The results shown in Fig. 2 indicate that the MQO activity, in contrast to the MDH activity, is highly dependent on the growth phase and that the activity decreases rapidly when the cells reach the stationary phase. The same observation was made for another strain (K-12 strain DSM 5698) and was also described earlier (12).

Properties of *mdh* and *mgo* deletion strains. To investigate the role of MQO and MDH in *E. coli*, deletions of the *mgo* and *mdh* genes were generated. The deletion of *mdh* causes a severe decrease in growth rates on all substrates except glucose (Fig. 3). Strain Δmdh does not grow on acetate or malate. Strain Δmgo appears to have no growth defect on all substrates tested. Nevertheless, the double mutant $\Delta mdh \Delta mgo$ grows more slowly on glucose, pyruvate, and lactate than does the

Δmdh strain. This effect was confirmed in several independent growth experiments (data not shown). These results show that deletion of the *mgo* gene has a phenotype although this phenotype is seen only in combination with a deletion of the *mdh* gene. Overexpression of *mgo* from a plasmid in the Δmdh strain was deleterious for growth (Table 3). It should be mentioned that the *mgo* gene is also expressed from the plasmid pECmqo in the absence of the inducer IPTG, which in the Δmgo strain leads to a twofold-higher MQO activity than that present in the wild type. Also, a deleterious effect of both the empty vector pET324 and pECmqo on the growth rate of *E. coli* MC4100 can be observed, which is relieved by the addition of IPTG. For this reason, the rate of growth with the *mgo* gene relative to that without the *mgo* gene is displayed. Increasing overexpression of *mgo* by increasing inducer concentration led to a decrease of the growth rate.

In accordance with the results concerning the growth rate, the deletion of *mgo* also did not affect the fitness in a competition experiment where MC4100 and Δmgo were cocultured. The fitness was tested by successive transfer of inoculum on minimal medium with glucose, acetate, or pyruvate, starting with a 1:1 proportion of wild-type and Δmgo cells (see Materials and Methods for details). The cultures were transferred every 24 h, so that each culture went through the lag, exponential, and stationary phases. After at least 50 generations, the wild-type and Δmgo strains were still present in a 1:1 proportion on all three substrates.

Effects of *sfcA*, *b2463*, and *pps* deletions on growth of *E. coli* wild type and Δmgo . In view of the standard free energies of the MDH and MQO reactions, it would be likely that under physiological conditions (pH 7.0) MDH catalyzes the reduction of oxaloacetate and MQO oxidizes malate to oxaloacetate. The results presented above show that, in contrast to the deletion of the *mdh* gene, the deletion of the *mgo* gene in *E. coli* has no discernible effect on growth. This observation does not rule out the possibility that MQO catalyzes malate oxidation, since an alternative pathway for the conversion of malate to oxaloacetate, formed by the collective action of malic enzyme, PEP synthase, and PEP carboxylase (15) (see Fig. 4), might take over this function from MQO in a Δmgo strain. Furthermore, assuming that MDH catalyzes oxaloacetate reduction, we present an alternative explanation for the deleterious effect of an *mdh* deletion in the Discussion. Clearly, the alternative route for malate oxidation depicted in Fig. 4 had to be blocked to further investigate the function of MQO.

E. coli contains a NAD-dependent and a NADP-depend-

TABLE 3. Growth rates of various strains with the vector pET324 or plasmid pECmqo, containing the *mgo* gene behind an IPTG-inducible promoter^a

Strain	IPTG concn (mM)	Growth rate (h ⁻¹)		Growth with <i>mgo</i> relative to growth without <i>mgo</i> (%)
		pET324	pECmqo	
MC4100	0	0.518 ± 0.003	0.512 ± 0.003	99 ± 1
	0.1	0.816 ± 0.010	0.284 ± 0.006	35 ± 4
	0.5	0.818 ± 0.012	0.194 ± 0.017	24 ± 10
Δmdh	0	0.563 ± 0.010	0.498 ± 0.009	89 ± 4
	0.1	0.817 ± 0.013	0.252 ± 0.013	31 ± 7
	0.5	0.807 ± 0.008	0.093 ± 0.014	12 ± 16
$\Delta mdh \Delta mgo$	0	0.513 ± 0.025	0.520 ± 0.008	101 ± 6
	0.1	0.634 ± 0.022	0.285 ± 0.017	45 ± 9
	0.5	0.622 ± 0.030	0.164 ± 0.015	26 ± 14

^a Growth rates and percentages are reported ± estimated standard deviations. The growth was measured on NMM with 0.5% glucose as carbon and energy source.

it is surprising that an *mgo* deletion in the wild type did not lead to an observable phenotype, and hence that no physiological function could be assigned to MQO.

In contrast, an *mdh* deletion induced severe growth defects. This observation per se does not imply that MDH oxidizes malate in the TCA cycle. The Gibbs standard free energy difference of the MDH reaction ($+28.6 \text{ kJ} \cdot \text{mol}^{-1}$) is very unfavorable for malate oxidation. The NADH/NAD ratio in *E. coli* varies from 0.75 under conditions of low oxygen pressure to 0.075 under high oxygen pressure (10). Under low oxygen pressure, the equilibrium oxaloacetate/malate ratio of the MDH reaction would then be 2×10^{-5} . In practice, this would mean that the concentration of malate in the cell should be at least 5 to 50 mM, assuming that the concentration of oxaloacetate should be at least 0.1 to 1 μM to meet the requirements of oxaloacetate-consuming enzymes. Such malate concentrations may be difficult to attain.

An alternative route for the oxidation of malate to oxaloacetate, involving malic enzyme and pyruvate decarboxylase, has been suggested previously to exist in *Bacillus subtilis* and has been named pyruvate shunt (11). This organism possesses an MDH and lacks an MQO. No gene similar to *mgo* was found in its genome, nor could MQO activity be demonstrated in isolated membrane fragments (D. Molenaar, unpublished results). It was suggested that in *B. subtilis* malate dehydrogenase reduces oxaloacetate and that, therefore, the pyruvate shunt is necessary for the production of oxaloacetate (11).

Since *E. coli* does not possess a pyruvate carboxylase and assuming, as seems to be the case in *C. glutamicum*, that MQO catalyzes malate oxidation while MDH reduces oxaloacetate, the absence of a phenotype for the *mgo* deletion mutant in *E. coli* might be due to a PEP shunt taking over its function (Fig. 4). MDH in this case would function as a (down-)regulator of the oxaloacetate concentration, and the effect of an *mdh* deletion might then be due to increased intracellular oxaloacetate concentrations. Oxaloacetate is known to be a strong inhibitor of key metabolic enzymes, e.g., succinate dehydrogenase (1), malic enzyme (K_i , 10 μM) (33), or PEP synthase (400 μM) (5). This might also be the reason why overexpression of *mgo* from a plasmid causes growth inhibition (Table 3), because it would disturb the balance between MDH and MQO activity and lead to an increased oxaloacetate concentration. The results in Table 4, however, indicate that the reason for the absence of a phenotype in a strain lacking MQO is not that a PEP shunt takes over the (hypothetical) role of MQO in the conversion of malate to oxaloacetate. Furthermore, since the $\Delta mgo \Delta pps$ and $\Delta mgo \Delta sfcA \Delta b2463$ strains lack the known alternative routes for conversion of malate to oxaloacetate, it has to be concluded that MDH oxidizes malate in *E. coli*. However, since the double mutant $\Delta mgo \Delta mdh$ still grows on lactate, pyruvate, and glucose, an alternative route has to exist to complete the TCA cycle.

An interesting phenomenon still to discuss is that the additional deletion of the *mgo* gene has a negative effect on the growth of the Δmdh strain when growing on glucose, pyruvate, or lactate (Fig. 3). The simplest interpretation of these results is that, in the absence of MDH, MQO is capable of sustaining a low TCA cycle activity. This activity is just enough to support a growth rate comparable to that of the wild type during growth on glucose, and it sustains somewhat lower growth rates on lactate or pyruvate. The capacity of MQO is not sufficient for growth on acetate or C_4 compounds, when a very high TCA cycle activity is required for the generation of metabolic energy. The fact that MQO completely takes over the function of MDH in the Δmdh strain during growth on glucose is contradictory to the assertion by others that the TCA cycle

is split during aerobic growth on glucose (13). Since MQO catalyzes irreversible oxidation of malate (17), it could not take over the function of an MDH which would reduce oxaloacetate in the reductive branch.

We are currently investigating possible reasons for the contrasting functions of MQO and MDH in *E. coli* and *C. glutamicum*. One of the obvious reasons to think of is the fact that there are significant differences in the specific activities of MQO and MDH and in the ratios of these activities between the two species. Compare, for example, the activities in Fig. 2 of this paper with those in Fig. 2 of the accompanying paper. Since the presence of both an MQO and MDH or their corresponding genes was observed in different eubacterial genera (6, 12, 16–18, 22, 23, 27), the results of these studies are of general importance for the physiology of the TCA cycle in bacteria.

ACKNOWLEDGMENTS

We thank R. P. Gunsalus, University of California at Los Angeles, for the gift of strains PC2 and PC35 and G. M. Church, Harvard Medical School, Boston, Mass., for the gift of plasmid pK03.

This research was funded by the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie in Germany (project 0316712).

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