

Malate:quinone oxidoreductase is essential for growth on ethanol or acetate in *Pseudomonas aeruginosa*

Utta Kretzschmar, Andreas Rückert, Jae-Hun Jeoung and Helmut Görisch

Author for correspondence: Helmut Görisch. Tel: +49 30 314 27582. Fax: +49 30 314 27581. e-mail: Goerisch@lb.TU-Berlin.de

Fachgebiet Technische Biochemie, Institut für Biotechnologie der Technischen Universität Berlin, Seestraße 13, D-13353 Berlin, Germany

***Pseudomonas aeruginosa* ATCC 17933 growing aerobically on ethanol uses a pyrroloquinoline quinone-dependent ethanol oxidation system. A mutant with an interrupted putative *mgo* gene, in which malate:quinone oxidoreductase (MQO), an enzyme involved in the citric acid cycle/glyoxylate cycle, was defective, showed a severe growth defect on ethanol and was unable to grow on acetate. Glucose, lactate, succinate or malate supported growth of the mutant. However, an NAD-dependent malate dehydrogenase activity could not be detected. Complementation of the mutant by the wild-type allele of the *mgo* gene restored wild-type behaviour. The wild-type expressed the dye-dependent MQO and NAD(P)-dependent malic enzymes (MEs). Pyruvate carboxylase (PC) was found upon growth of the wild-type and the mutant on all substrates studied. PC activity in the wild-type was induced on glucose and lactate and was always higher on all substrates in the *mgo* mutant. In *P. aeruginosa* ATCC 17933, an active MQO is required for growth on ethanol or acetate, while with glucose, lactate, succinate or malate an apparent bypass route operates, with MEs using malate for generating pyruvate, which is carboxylated to oxaloacetate by PC. To the authors' knowledge, this is the first time that a specific mutant MQO phenotype has been observed, caused by the inactivation of a gene encoding MQO activity. *mgo* of *P. aeruginosa* ATCC 17933 corresponds to *mgoB* (PA4640) of the *P. aeruginosa* PAO1 genome project.**

Keywords: ethanol oxidation, malate conversion, pyruvate carboxylase, malic enzyme

INTRODUCTION

A number of Gram-negative bacteria synthesize enzymes with pyrroloquinoline quinone (PQQ) as cofactor when grown on glucose or various alcohols. These PQQ-dependent quinoprotein aldose and alcohol dehydrogenases are located in the periplasm. *Pseudomonas aeruginosa* ATCC 17933 grows aerobically with ethanol as sole carbon and energy source. We have previously identified an *exaABC* gene cluster that encodes a

quinoprotein ethanol dehydrogenase (*exaA*), an NAD-dependent acetaldehyde dehydrogenase (*exaC*) and a cytochrome c_{550} (*exaB*) (Diehl *et al.*, 1998; Reichmann & Görisch, 1993; Schobert & Görisch, 1999) (Fig. 1). *exaDE* encode a sensor kinase (ExaD) and a response regulator (ExaE), which direct transcription of *exaA* but not of *exaBC* (Schobert & Görisch, 2001).

Schobert & Görisch (1999) isolated a number of mutants of *P. aeruginosa* ATCC 17933 that were unable to grow on ethanol. Recently, we demonstrated that in *P. aeruginosa* ATCC 17933 an acetyl-CoA synthetase, encoded by *acsA*, is essential for growth on ethanol (Kretzschmar *et al.*, 2001). In the present study, we have identified the *mgo* gene, encoding a malate:quinone oxidoreductase (MQO), which is also essential in *P. aeruginosa* for growth on ethanol or acetate.

Abbreviations: MDH, malate dehydrogenase; ME, malic enzyme; MQO, malate:quinone oxidoreductase; PC, pyruvate carboxylase; PEP, phosphoenolpyruvate; PEP-C, PEP carboxylase; PQQ, pyrroloquinoline quinone.

The GenBank accession number for the *Pseudomonas aeruginosa* ATCC 17933 *mgo* sequence reported in this work is AY129296.

Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant properties	Reference
Strain		
<i>P. aeruginosa</i> ATCC 17933	Wild-type	Cetin <i>et al.</i> (1965)
<i>P. aeruginosa</i> MS7	ATCC 17933 derivative, mutant class I	Schobert & Görisch (1999)
<i>P. aeruginosa</i> UK2	ATCC 17933 derivative, <i>mgo</i> ::Km ^r	This study
<i>E. coli</i> JM109	F' <i>traD36 lac^a Δ(lacZ) M15 proA⁺B⁺/recA1 endA1 gryA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>E. coli</i> HB101	<i>supE44 hsdS20 (r_B⁻ m_B⁻) recA13 ara-14 proA2 lacY1 galk2 rpsL20 xyl-5 mtl-1</i>	Boyer & Roulland-Dussoix (1969)
<i>E. coli</i> DH5α	<i>supE44 ΔlacU169(φ80 lacZΔM15) hsdR17 recA1 gyrA96 thi-1 relA1</i>	Hanahan (1983)
Plasmid		
pLAFR3	Tc ^r ; broad-host-range cosmid	Staskawicz <i>et al.</i> (1987)
pRK2013	Km ^r ; helper plasmid for triparental mating	Figurski & Helinski (1979)
pSUP1021	Tc ^r Cm ^r Km ^r ; suicide vector containing Tn5	Simon <i>et al.</i> (1986)
pUC19	Ap ^r ; cloning and expression vector	Yanisch-Perron <i>et al.</i> (1985)
pUCP20T	Ap ^r ; broad-host-range plasmid	Schweizer <i>et al.</i> (1996)
pTB3021	Tc ^r ; cosmid complementing mutant MS7; 25.7 kb genomic DNA partially digested with <i>Sau3AI</i> from <i>P. aeruginosa</i> and cloned into <i>BamHI</i> site of pLAFR3	Schobert & Görisch (1999)
pTB3131	Ap ^r Km ^r ; 1.0 kb PCR product with promoter and kanamycin-resistance gene of Tn5 cloned between the <i>EcoRI</i> and <i>BamHI</i> sites of pUC19	Kretzschmar <i>et al.</i> (2001)
pTB4109	Ap ^r ; 7.5 kb <i>EcoRI</i> fragment from pTB3021 cloned between <i>EcoRI</i> sites of pUCP20T	This study
pTB4110	Ap ^r ; 4.2 kb <i>PaeI</i> fragment from pTB4109 cloned between <i>PaeI</i> sites of pUCP20T	This study
pTB4111	Ap ^r ; 2.1 kb, PCR product with complete <i>mgo</i> cloned between the <i>BamHI</i> and <i>EcoRI</i> sites of pUCP20T (<i>mgo</i> orientation same as P _{<i>lac</i>} of pUCP20T)	This study
pTB4112	Ap ^r Km ^r ; 1.0 kb <i>SmaI</i> - <i>SmaI</i> fragment from pTB3131 containing Km ^r gene of Tn5 cloned in <i>SmaI</i> site of pTB4111 (kanamycin-resistance gene in same orientation as <i>mgo</i>)	This study
pTB4116	Ap ^r Km ^r ; 3.1 kb <i>EcoRI</i> - <i>HindIII</i> fragment from pTB4112 cloned between the <i>EcoRI</i> and <i>HindIII</i> sites of pUC19	This study

Tc^r, tetracycline-resistant; Km^r, kanamycin-resistant; Ap^r, ampicillin-resistant; Cm^r, chloramphenicol-resistant.

transform *P. aeruginosa* ATCC 17933 by electroporation (Smith & Iglewski, 1989). Potential site-directed double-crossover mutants with a kanamycin-resistant phenotype were selected for loss of ampicillin resistance.

Preparation of cell-free extracts and membrane fractions. All operations were done at 4 °C. Bacteria were grown to late-exponential phase, harvested, washed twice with ice-cold 0.1 M Tris/HCl buffer (pH 7.4) containing 10 mM MgSO₄, 10% glycerol and 5 mM β-mercaptoethanol (Saeki *et al.*, 1999), and resuspended in the same buffer. After cell disruption by sonication, cell debris was removed by centrifugation for 30 min at 6000 g. Part of the supernatant was used as the cell-free extract; the other part was used to obtain the membrane fraction. The supernatant was centrifuged for 1 h at 100 000 g; the resulting membrane-rich pellet was washed with ice-cold 50 mM HEPES buffer (pH 7.5) containing 10 mM potassium acetate, 10 mM CaCl₂ and 5 mM MgCl₂ (Molenaar *et al.*, 1998), before being resuspended in a small volume of the HEPES buffer. Cell-free extracts and membrane fractions were split into aliquots and either assayed immediately for enzymic activity or stored at -20 °C. The protein content of the cell-free extracts was between 2 and 5 mg protein ml⁻¹,

while the membrane fractions contained between 9 and 20 mg protein ml⁻¹.

PQQ determination. PQQ in the supernatants of *P. aeruginosa* cultures was determined according to Geiger & Görisch (1987). Crude membranes of *E. coli* containing the apoprotein of quinoprotein glucose dehydrogenase were used to reconstitute holoenzyme activity.

Enzyme assays. All assays were carried out at 25 °C in a total volume of 1 ml. The MQO activity was assayed with the membrane fraction. The activity was determined by measuring the decrease in absorbance of 2,6-dichlorophenol indophenol (DCPIP) at 600 nm, assuming an absorption coefficient of 22 cm⁻¹ mM⁻¹ (Armstrong, 1964). The test of O'Brien & Taylor (1977) was modified and contained, in a total volume of 1 ml, 50 mM Tris/HCl, 0.05 mM DCPIP, 0.1 mM phenazine methosulfate, 5 mM ethylamine, 5 mM KCN, 10 μM FAD and 1 mM L-malate. All other enzyme activities were determined in cell-free extracts. NAD-dependent MDH activity was determined using oxaloacetate as the substrate in a mixture containing 0.1 M Tris/HCl buffer (pH 7.5, with 0.06% BSA), 15 mM KCN, 0.2 mM NADH and 1 mM oxaloacetate. Catalytic activity was calculated from the

decrease in absorbance of NADH at 340 nm, using an absorption coefficient of $6.22 \text{ cm}^{-1} \text{ mM}^{-1}$ (Horecker & Kornberg, 1948). Pyruvate carboxylase (PC) and PEP carboxylase (PEP-C) activities were measured according to Saeki *et al.* (1999) by the decrease in absorbance of NADH at 340 nm in a coupled test with MDH. The reaction was started by the addition of pyruvate or phosphoenolpyruvate (PEP). The activity of malic enzyme (ME) was determined by the increase in absorbance due to the reduction of NAD. The method of Bergmeyer & Graßl (1987) was used with some modifications. The reaction mixture contained 0.1 M Tris/HCl buffer (pH 7.5), 2 mM NAD (or NADP), 5 mM L-malate and 4 mM MnCl_2 . The reaction was started by the addition of MnCl_2 . Specific activities of all of the enzymes assayed were expressed as $\mu\text{mol product formed min}^{-1} (\text{mg protein})^{-1}$. Using the same sample, standard deviations of the enzyme assays were calculated as $\pm 5.6\%$ for MQO, $\pm 4.2\%$ for PC, $\pm 2.6\%$ for ME (NAD) and $\pm 15.5\%$ for ME (NADP). Protein concentrations were determined using the method of Groves *et al.* (1968).

Internet tools. BLAST was used for DNA or protein database searches (Altschul *et al.*, 1997), the advanced BLAST2 server search of the NCBI (<http://www.ncbi.nlm.nih.gov/>) was used for comparing the sequence of *mgo* from *P. aeruginosa* ATCC 17933 to *mgoB* from *P. aeruginosa* PAO1, and the *Pseudomonas aeruginosa* Genome Database (<http://pseudomonas.bit.uq.edu.au/>; version from 21 November 2001) was used to obtain the DNA sequence of the PAO1 *mgoB* gene.

RESULTS AND DISCUSSION

Characteristics of mutant MS7

The chemical mutant MS7 of *P. aeruginosa* ATCC 17933 (Schobert & Görisch, 1999) was grown in LB broth and inoculated into minimal medium containing

different carbon sources (see Methods). In contrast to wild-type cells, the mutant cells showed a severe growth defect on ethanol and were unable to grow on 2,3-butanediol or acetate. However, when induced on ethanol, the mutant possessed quinoprotein ethanol dehydrogenase (QEDH) activity and excreted PQQ into the culture medium. QEDH activity and the levels of extracellular PQQ found with the mutant were similar to the values found when the wild-type was grown on ethanol. Growth of mutant MS7 on glucose was almost comparable to that of the wild-type; succinate, fumarate and malate also supported growth of the mutant. However, with the latter substrates, the lag phase of the mutant was prolonged and its growth rate was reduced (Table 2).

Subcloning of an *mgo* gene and complementation of mutant MS7

pTB3021, from a cosmid gene library of *P. aeruginosa* ATCC 17933, complemented mutant MS7 and restored growth of the organism on ethanol. We sequenced 250 bp from the 5' and 3' ends of the approximately 24 kb insert of pTB3021. The DNA sequences of *P. aeruginosa* ATCC 17933 and *P. aeruginosa* PAO1 differ by only 2% (Kretzschmar *et al.*, 2001). To evaluate the relevant sequence of strain ATCC 17933, we used the published PAO1 sequence, which turned out to be 25.7 kb long. This sequence information was used to generate subclones. Plasmids pTB4109 and pTB4110, with inserts of 7.5 and 4.2 kb, respectively, both derived from the cosmid insert, restored growth to mutant MS7. The insert of pTB4110 carried five ORFs. Four of these ORFs corresponded to genes *PA4641-PA4644*, which

Table 2. Growth of *P. aeruginosa mgo* mutants on various substrates

Growth within 10 h was recorded as the final OD_{620} value when reaching stationary phase. ND, Not determined; + + +, OD_{620} value of between 0.9 and 1.2; + +, OD_{620} value of between 0.6 and 0.9; +, OD_{620} value of between 0.3 and 0.6; -, no growth; (+ + +), as for + + + but indicates a prolonged lag phase and a reduced growth rate compared to the wild-type. Substrate concentrations used are as indicated in Methods.

Substrate	Wild-type	Mutant				
		MS7	UK2	Complemented by		
				pTB4109		pTB4111
				MS7	MS7	UK2
Glucose	+ + +	+ + +	+ + +	ND	ND	ND
Ethanol	+ + +	-	-*	+ + +	+ + +	+ + +
2,3-Butanediol	+ + +	-	-	ND	ND	ND
Acetate	+ +	-	-	ND	ND	+ +
Succinate	+ + +	(+ + +)	(+ + +)	+ + +	+ + +	+ + +
Fumarate	+ + +	(+ + +)	(+ + +)	ND	ND	ND
Malate	+ + +	(+ + +)	(+ + +)	ND	ND	ND
Pyruvate	+	+	+	ND	ND	ND
Lactate	+ + +	ND	+ + +	ND	ND	ND

* When growth was recorded for 33 h, slow growth was observed starting after about 15 h and continuing up to 33 h.

code for unknown functions. ORF1 corresponded to PA4640, the deduced amino acid sequence of which shows high similarity to bacterial MQOs.

A clone carrying only the intact ORF1 from strain ATCC 17933 was obtained by PCR. In pTB4111, ORF1 was oriented linear with the *lac* promoter of pUCP20T, and complementation of mutant MS7 with this clone restored growth of this organism on ethanol. To confirm that complementation occurred *in trans* and was not a result of homologous recombination, plasmid DNA was prepared from complemented mutant strains and used to transform *E. coli* JM109. The resulting transformants were used again in triparental matings and successfully complemented the original mutant, MS7.

pTB4110 was used for sequencing both strands of the putative *mgo* gene of strain ATCC 17933. The gene was 1524 bp long, and its deduced amino acid sequence showed high similarity (67%) to the MQOs from *C. glutamicum* (Molenaar *et al.*, 1998), *E. coli* (van der Rest *et al.*, 2000) and *Neisseria meningitidis* (Leighton *et al.*, 2001). A putative FAD-binding site, DMLLVGAG, near the N-terminal region of the protein was also found. This sequence was almost identical to the conserved motif DhhhhG(A/G)G found in *C. glutamicum* and *E. coli* MQOs (Molenaar *et al.*, 1998), where h stands for the amino acid I, V or L. In addition, the N terminus of the putative protein contained the motif GXGXXG (GTGHAG) of the ADP-binding $\beta\alpha\beta$ fold, which was found in the MQO of *H. pylori* (Kather *et al.*, 2000) and in 16 other flavin-binding enzymes (Austin & Larson, 1991) but not in the MQO of *C. glutamicum* (Molenaar *et al.*, 1998).

A putative Shine–Dalgarno sequence (Rothmel *et al.*, 1991), GGAGA, was found 7 bp upstream of the *mgo* start codon ATG. A putative *rho*-independent transcription terminator was found 11 bp downstream of the translation stop codon TGA. The inverted repeat had a 12 bp stem. The gene corresponding to ORF1 from strain ATCC 17933 in the *P. aeruginosa* PAO1 genome database (PA4640) is designated *mgoB*. A comparison of the putative *mgoB* gene with the ORF1 sequence from *P. aeruginosa* ATCC 17933 revealed there to be only five base pairs different between the two sequences (0.3% divergence). These changes did not cause differences between the deduced amino acid sequences of the two Mgo proteins.

Inactivation of *mgo*

The product of *mgo* is essential for growth of *P. aeruginosa* on ethanol or acetate, as demonstrated by site-directed mutagenesis using pTB4116. A kanamycin-resistant insertion mutant (UK2) was isolated, as described in Methods. Mutant UK2, like mutant MS7, showed a severe growth defect on ethanol and was unable to grow on 2,3-butanediol or acetate. PCR with the genomic DNA of mutant UK2 confirmed the presence of the kanamycin-resistance cassette in *mgo* (*mgo*::Km^r) (data not shown). The kanamycin-resistance gene was transcribed in the same orientation

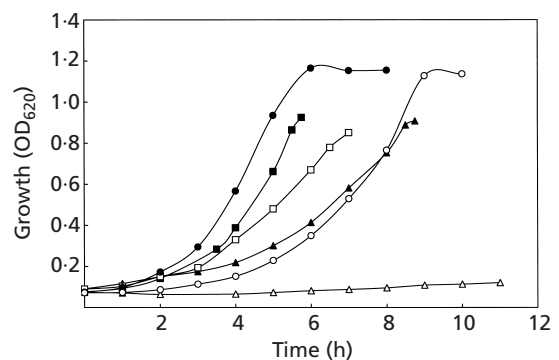


Fig. 2. Aerobic growth of wild-type *P. aeruginosa* (solid symbols) and the *mgo* mutant UK2 (open symbols) in minimal medium containing (▲, △) ethanol, (■, □) glucose or (●, ○) succinate. Substrate concentrations are as indicated in Methods.

as *mgo*. The *mgo*::Km^r allele in mutant UK2 did not express a toxic variant of MQO, since complementation with pTB4111, carrying only *mgo*, restored wild-type growth on ethanol, acetate and succinate (Table 2).

Characteristics of mutant UK2

Mutant UK2 (*mgo*::Km^r) did not grow on ethanol within 10 h incubation, whereas wild-type cells and UK2 cells complemented with pTB4111 reached stationary phase after 8–9 h incubation (Fig. 2). However, after 15 h incubation mutant UK2 started to grow very slowly. After 33 h incubation, mutant UK2 reached an optical density close to that observed for the wild-type after 9 h incubation. The observed growth was not caused by spontaneous revertants, because when this UK2 culture was used as the inoculum for a subsequent cultivation the long lag phase and very slow growth were observed again.

When inoculated into medium containing acetate, no growth of mutant UK2 was detected within 50 h incubation, but complementation of the mutant with pTB4111 restored the wild-type behaviour.

Growth of mutant UK2 on glucose, lactate and pyruvate was almost comparable to that of the wild-type. Also, succinate, fumarate and malate supported growth of mutant UK2, but its lag phase and generation time were increased with respect to the wild-type (Table 2; Fig. 2).

Activities of malate-converting and oxaloacetate-forming enzymes

Wild-type *P. aeruginosa*, as well as mutant UK2, was grown on different carbon sources. Cells were collected by centrifugation at the late-exponential phase and disrupted by sonication. The soluble cytoplasmic and periplasmic fractions and the supernatant after removal of membrane fragments by ultracentrifugation at 100 000 g, as well as the membrane fraction, were tested for their MQO, MDH, ME, PC and PEP-C activities. The MQO activity was associated with the membrane

Table 3. Activities of malate-converting and oxaloacetate-forming enzymes in the wild-type and the *mgo*::Km^r mutant UK2 of *P. aeruginosa* after growth on different carbon sources

MQO activity was determined in the membrane fraction. ME, PC and PEP-C activities were determined in cell-free extracts. The results are shown as the means of two independent experiments, except where indicated.

Carbon source	Strain	MQO [mU (mg protein) ⁻¹]	ME [mU (mg protein) ⁻¹]		PC [mU (mg protein) ⁻¹]	PEP-C [mU (mg protein) ⁻¹]
			NAD	NADP		
Ethanol	Wild-type	84 (±10)†	31 (±3)†	8 (±1)†	11 (±2)†	7
	Mutant*	9 (±2)†	37 (±14)†	21 (±4)†	57 (±7)†	8
Glucose	Wild-type	107 (±23)†	33 (±4)†	19 (±15)†	41 (±3)†	7
	Mutant	25 (±6)†	42 (±9)†	34 (±17)†	66 (±10)†	6
Succinate	Wild-type	105 (±32)†	29 (±3)†	29 (±12)†	12 (±0.4)†	6
	Mutant	22 (±6)†	51	21	51 (±6)†	ND
Acetate	Wild-type	121	28	5	17	5
Lactate	Wild-type	94	31	22	39	ND
	Mutant	14	36	21	48	ND
Malate	Wild-type	88	35	32	6	ND
	Mutant	7	43	23	52	ND

* Determined after very slow growth for 33 h.

† These values are means calculated from three separately grown cell batches; ±SD values are shown in parentheses.

fraction, indicating that it is a membrane-bound or membrane-associated enzyme. ME, PC and PEP-C appeared to be soluble enzymes. The activities of the different enzymes are shown in Table 3.

The gene from strain ATCC 17933 that had high similarity to bacterial MQOs did indeed encode an enzyme with MQO activity (EC 1.1.99.16), as demonstrated by the data shown in Table 3. The membrane fraction of the wild-type cells showed MQO activity of between 80 and 120 mU (mg protein)⁻¹, independent of the carbon source. However, the membrane fraction from the mutant UK2 cells showed only a very low activity. This residual activity may be due to a second *mgo* gene, recorded in the *P. aeruginosa* PAO1 genome database and referred to as *mgoA*. After growth of the organism on acetate medium, cell-free extracts of mutant UK2 transformed by triparental mating with pTB4111 showed a more than twofold higher specific activity of MQO [43 mU (mg protein)⁻¹] than extracts from wild-type cells grown under the same conditions [17 mU (mg protein)⁻¹].

In contrast to *P. aeruginosa*, MQO activity in *C. glutamicum* and *E. coli* is regulated in response to the carbon source used, with the activity of the enzyme being about fourfold higher on acetate than on glucose. MQO activity in *C. glutamicum* varied between 110 mU (mg protein)⁻¹ on glucose and 370 mU (mg protein)⁻¹ on acetate (Molenaar *et al.*, 2000), while in *E. coli* on glucose 12 mU (mg protein)⁻¹ were found compared to 47 mU (mg protein)⁻¹ on acetate (van der Rest *et al.*, 2000).

A probable L-MDH (EC 1.1.1.37) gene is recorded in the *P. aeruginosa* PAO1 genome database (gene

PA1252). However, neither wild-type nor mutant UK2 cells of *P. aeruginosa* ATCC 17933 showed any NAD-dependent L-MDH activity under any of the conditions tested. Also, no L-MDH activity was found with NADP. Commercial L-MDH (EC 1.1.1.37) and cell-free extracts from *Rhodococcus* strain DTB (Hauck *et al.*, 2001) were used as a positive control for enzyme activity. Hopper *et al.* (1970) have already noted the absence of any NAD- or NADP-dependent L-MDH activity in a strain of *Pseudomonas*; in addition, these authors also found a dye-dependent, membrane-bound L-MDH activity in the same strain.

It appears that *P. aeruginosa* 17933 contains two MEs – one that lacks an absolute coenzyme specificity but is more active with NAD than with NADP (EC 1.1.1.39; MDH, decarboxylating), and one that is highly specific for NADP (EC 1.1.1.40; MDH, oxaloacetate-decarboxylating). This is in agreement with the *P. aeruginosa* PAO1 genome database, where a gene encoding ME (PA5046) and a gene encoding a probable ME (PA3471) are recorded. We found an inhibition of the NAD-dependent ME by acetyl-CoA. With 0.2 mM acetyl-CoA, the initial activity was reduced to 70%, and to 40 and 20%, respectively, with 2 and 10 mM acetyl-CoA. In contrast, PC was not inhibited by 0.2–10 mM acetate nor by 0.5–2.5 mM acetyl-CoA. Also, the NADP-dependent ME activity was not inhibited by acetyl-CoA up to a concentration of 4 mM, and acetate did not show any regulatory effect.

The reaction catalysed by the NADP-dependent enzyme was reversible, since a reductive carboxylation of pyruvate could be detected (data not shown). NAD- and NADP-dependent ME activities did not vary significantly with different carbon sources. Mutant UK2

showed a small increase in NAD- and NADP-dependent ME activities.

PC activity (EC 6.4.1.1) in wild-type cells was strongly influenced by the carbon source used for growth. On glucose and lactate, the specific activity of this enzyme was induced three- to fourfold, compared to growth on ethanol, acetate or succinate. In mutant UK2, the specific activity of PC increased four- to eightfold on all carbon sources tested, except for on glucose and lactate, where it increased about 1.5-fold when compared to the wild-type.

A substrate-inducible PC, with highest activity observed in cells grown on glucose or lactate, has already been described for *Pseudomonas citronellolis* (O'Brien *et al.*, 1977). Roehl *et al.* (1983) also reported a PC, and the gene encoding this protein, in *P. aeruginosa* PAO1.

PC is an inducible, anaplerotic enzyme that is essential for the utilization of all carbohydrates. In *C. glutamicum*, besides PC, PEP-C is also needed for growth on lactate or glucose to replenish the TCA cycle with precursors for biosynthesis. In this bacterium, the formation of PC is regulated by the carbon source used and the activity of the enzyme is inhibited by 0.1 mM acetyl-CoA (Peters-Wendisch *et al.*, 1997; Wendisch *et al.*, 2000).

In *P. aeruginosa* ATCC 17933, only low constitutive PEP-C (EC 4.1.1.31) activity could be detected on all of the carbon sources tested. The enzyme appeared to be uninducible (Table 3).

Proposed alternative pathway for malate oxidation

Oxaloacetate is an important precursor for gluconeogenesis and the synthesis of amino acids. It is also the substrate of citrate synthase in the citric acid cycle. Since in the wild-type and the mutant UK2 no NAD-dependent MDH activity could be detected, MQO must be the enzyme responsible for malate oxidation. However, the mutant lacking the *mgo* gene product was able to grow on glucose and lactate at levels similar to those of the wild-type; it was also able to grow on succinate, fumarate and malate, albeit with a prolonged lag phase and a reduced growth rate. We assume that in mutant UK2 an alternative pathway formed by the collective actions of ME, generating pyruvate, and PC, generating oxaloacetate, takes over the function of MQO (Fig. 1). Indeed, PC activity was induced about fivefold in the mutant (Table 3).

Also, for *B. subtilis*, which possesses an MDH and lacks MQO, a pyruvate shunt has been described (Diesterhaft & Freese, 1973). ME and PC form a bypass to the citric acid cycle during growth of this organism on malate, to allow the production of sufficient oxaloacetate for gluconeogenesis and biosynthesis. In contrast to the situation in *P. aeruginosa*, the activity of PC was not dependent on the growth medium and the enzyme was strongly activated by acetyl-CoA (Diesterhaft & Freese, 1973). In *E. coli*, which contains MQO and MDH, MQO partly takes over the function of MDH in an *mdh*

mutant. However, an *mgo* mutant did not show a specific phenotypic defect (van der Rest *et al.*, 2000).

The effects of growth substrates on the specific activities of MEs and the response of enzyme activity to effector molecules reported in the literature differ between different organisms. For instance, in *Rhizobium meliloti*, which contains two MEs, the NAD(P)-dependent ME showed allosteric regulation by acetyl-CoA. The activity of this enzyme was reduced to 2% by 0.1 mM acetyl-CoA. A strictly NADP-specific ME was not subjected to regulatory mechanisms (Voegele *et al.*, 1999).

In our *mgo* mutant, UK2, we assumed that a bypass for the oxidation of malate to oxaloacetate was operating through the activities of a NAD-dependent ME and a NADP-dependent ME, together with a higher expression of PC activity. With glucose, lactate or pyruvate as the carbon source, growth of the mutant UK2 was only slightly impaired with respect to the wild-type. Pyruvate is available in abundance and with the increased activity of PC enough oxaloacetate can be synthesized for biosynthetic purposes, and for gluconeogenesis in the case of either lactate or pyruvate as the carbon source. Under these growth conditions, the activities of the MEs are only necessary to close the citric acid cycle for generating energy.

When mutant UK2 was grown on succinate or malate, the complete bypass of the citric acid cycle with ME and PC activities was needed to obtain energy, as well as to provide oxaloacetate for gluconeogenesis and amino acid biosynthesis. Under these conditions, the bypass seemed to operate at its limits, since growth of mutant UK2 was strongly delayed and the growth rate was reduced compared to that of the wild-type.

Growth of wild-type cells on ethanol or acetate is a strenuous exercise, because more ATP is required for the synthesis of cell material than when the same cells are grown on succinate or glucose. Under these conditions, even for wild-type cells the lag phase is increased and the growth rate is reduced compared to that on the two other carbon sources (Fig. 2). Mutant UK2 does not grow at all on ethanol or acetate within 10 h incubation. On these carbon sources, the bypass of the citric acid cycle formed by ME and PC activities would again be needed to allow operation of the citric acid cycle for the generation of energy; this bypass would also be required to supply intermediates for both gluconeogenesis and biosynthetic purposes. Apparently, the capacity of the bypass is not sufficient to support growth on ethanol or acetate, resulting in energetic limitations or a limitation in a biosynthetic precursor. In addition, growth on ethanol or acetate requires all of the carbon used in metabolism to be provided as acetyl-CoA. We assume that under these conditions the acetyl-CoA concentration would be raised to levels higher than those of mutant UK2 grown on malate or glucose; hence, the activity of the NAD-dependent ME would be inhibited, lowering the capacity of the bypass even further.

However, to prove the operation of the proposed pathway, the generation of mutants with defects in ME,

PC and PEP carboxykinase, PEP-C or PEP synthase and in combination with a defective MQO will be necessary, together with enzymic studies with respect to the induction and regulatory properties of the various enzymes. These studies are under way in our laboratory.

Concluding remarks

The reported results demonstrate that the putative *mgo* gene of *P. aeruginosa* ATCC 17933 encodes an MQO activity. This gene corresponds to *mgoB* (PA4640) of the *P. aeruginosa* PAO1 genome database. The enzyme, which catalyses the conversion of malate to oxaloacetate in the citric acid cycle, has so far not been studied in *P. aeruginosa*. A sufficient level of MQO activity is essential for growth of *P. aeruginosa* on ethanol or acetate. However, the *mgo* mutant UK2 can grow on glucose, lactate and pyruvate, and also on fumarate, succinate and malate, apparently by the collective actions of ME and PC, which bypass the MQO-catalysed conversion of malate to oxaloacetate.

The residual low MQO activity found in the membrane fraction of mutant UK2, in which *mgo* was interrupted by a kanamycin-resistance cassette, may have been caused by a second putative *mgo* gene, *mgoA* (PA3452), found in the genome of *P. aeruginosa* PAO1.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft.

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Received 5 July 2002; revised 12 August 2002; accepted 11 September 2002.