Suppression of *Escherichia coli* formate hydrogenlyase activity by trimethylamine *N*-oxide is due to drainage of the inducer formate

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The effect of the addition of trimethylamine *N*-oxide (TMAO) in the growth medium on *Escherichia coli* anaerobic fermentative and respiratory pathways was examined. Formate dehydrogenase H (FDH-H) activity was totally repressed by the addition of 40 mM TMAO, whereas the overall hydrogenase (HYD) activity was reduced by 25%. Accordingly, expression of *lacZ* operon fusions with the *fdhF* and *hycB* structural genes specifying FDH-H and HYD3 was reduced sevenfold and eightfold, respectively, leading to suppression of an active formate hydrogenlyase system. In contrast, global respiratory formate-dependent phenazine methosulphate reductase (FDH-PMS) activity, which consists of both the major anaerobic FDH-N enzyme and the aerobic FDH-Z isoenzyme, was increased approximately twofold. This was corroborated by a 2.5-fold stimulation of the sole *fdoG-uidA* transcriptional fusion which reflects the synthesis of the respiratory aerobic FDH-Z enzyme. In *fdhD*, *fdhE* or *torA* mutants lacking either FDH-PMS activity or TMAO reductase (TOR) activity, the formate hydrogenlyase pathway was no longer inhibited by TMAO. In addition, introduction of 30 mM formate in the growth medium was found to relieve the repressive effect of TMAO in the wild-type strain. When TMAO was added as terminal electron acceptor a significant enhancement of anaerobic growth was observed with the wild-type strain and the *fdoG* mutant. It was associated with the concomitant suppression of the formate hydrogenlyase enzymes. This was in contrast to the *fdnG* and *torA* mutants whose growth pattern and fermentative enzymes remained unaffected. Taken together, these results strongly suggest that formate-dependent reduction of TMAO via FDH-N and TOR reduces the amount of formate available for induction of the formate hydrogenlyase pathway.

Keywords: *Escherichia coli* K-12, anaerobiosis, trimethylamine *N*-oxide, formate hydrogenlyase, gene expression

INTRODUCTION

The facultative anaerobe *Escherichia coli* exhibits a remarkable flexibility to adapt to different growth conditions and to derive energy from anaerobic growth

using a variety of electron transfer processes. Organic substrates are oxidized by passing the electrons to any of a number of terminal electron acceptors like nitrate and trimethylamine *N*-oxide (TMAO) (Gennis & Stewart, 1996). Formate produced from the anaerobic cleavage of

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Abbreviations: FDH-H, hydrogen-linked formate dehydrogenase; FDH-N, nitrate-induced anaerobic formate dehydrogenase; FDH-PMS, formate-dependent phenazine methosulphate reductase; FDH-Z, aerobic formate dehydrogenase; HYD, hydrogenase; NAR-A, nitrate-induced anaerobic terminal nitrate reductase; NAR-Z, aerobic nitrate reductase; TMAO, trimethylamine *N*-oxide; TOR, trimethylamine *N*-oxide reductase; TYEP, Tryptone Yeast Extract Phosphate.

pyruvate by pyruvate-formate lyase can be metabolized by distinct routes. In the absence of exogenous electron acceptors, it is converted to carbon dioxide and dihydrogen by the fermentative formate hydrogenlyase complex. This pathway located on the inner side of the cytoplasmic membrane includes a formate dehydrogenase called FDH-H (H for hydrogen), one of the three hydrogenase isoenzymes synthesized in anaerobic cells, termed HYD3, and two electron carriers (Böck & Sawers, 1996). The *fdhF* structural gene encodes the FDH-H selenopolypeptide (Zinoni *et al.*, 1986) and the *hyc* operon directs the synthesis of nine gene products which include the other structural components of the formate hydrogenlyase complex (Sauter *et al.*, 1992; Rossmann *et al.*, 1995). Expression of these genes is inducible under fermentative growth conditions and repressed by oxygen and nitrate. Transcription is activated by the transcriptional regulator FhlA which responds to the intracellular concentration of formate (Rossmann *et al.*, 1991).

In the presence of nitrate, the membrane-bound formate-nitrate respiratory chain is induced. This proton-translocating pathway is constituted by a second formate dehydrogenase (FDH-N, N for nitrate) and a nitrate-induced anaerobic terminal nitrate reductase (NAR-A) linked by a quinone (Gennis & Stewart, 1996). The *fdnGHI* and *narGHJI* operons encoding FDH-N and NAR-A, respectively, have been cloned and sequenced (Berg *et al.*, 1991; Sodergren & DeMoss, 1988; Blasco *et al.*, 1989). Studies of transcriptional regulation have established that their expression is dependent on anaerobiosis and nitrate but not on formate (Stewart, 1993).

A second formate-nitrate respiratory pathway has been subsequently identified, which is composed of two immunologically related isoenzymes of FDH-N and NAR-A, termed FDH-Z and NAR-Z, respectively (Pommier *et al.*, 1992; Iobbi-Nivol *et al.*, 1990). The corresponding *fdoGHI* and *narZYWV* structural genes have been isolated and found to bear strong identity (around 70%) with the *fdnGHI* and *narGHJI* operons (Plunkett *et al.*, 1993; Blasco *et al.*, 1990). However, in contrast to the major anaerobic system, this second respiratory chain is produced irrespective of the presence of oxygen and barely induced by nitrate (Iobbi-Nivol *et al.*, 1990; Abaibou *et al.*, 1995). It has been proposed that such a pathway would allow the cell to adapt rapidly to anaerobiosis after a sudden shift from aerobic to anaerobic conditions in the presence of nitrate (Abaibou *et al.*, 1995). Two other genes, *fdhD* and *fdhE*, located in the close vicinity of the *fdoGHI* cluster, are likely to act post-translationally in the formation of both active respiratory FDH enzymes (Schlindwein *et al.*, 1990; Pommier *et al.*, 1992; Stewart *et al.*, 1991).

TMAO can also be used by *E. coli* as electron acceptor for anaerobic respiration (Barrett & Kwan, 1985). Reduction of TMAO is catalysed by two well-characterized molybdoenzymes, a membrane-bound constitutive enzyme, called DMSO reductase and a periplasmic inducible enzyme, called TMAO reductase

(TOR) (Weiner *et al.*, 1988; Silvestro *et al.*, 1989). In both cases, enzyme synthesis requires anaerobic growth conditions, but whereas expression of DMSO reductase and the corresponding *dmsABC* operon is repressed by nitrate, that of TOR and the relevant *torCAD* operon is not subjected to this hierarchical control (Bilous & Weiner, 1988; Pascal *et al.*, 1984). Recently TMAO induction of *torCAD* operon expression has been demonstrated to be mediated via a two-component system, including the response regulator protein TorR and the sensor protein TorS, and able to interact with a periplasmic inducer-binding protein called TorT (Simon *et al.*, 1994; Jourlin *et al.*, 1996a, b).

Expression of the anaerobic electron transport pathways in response to the energetically more favourable substrate nitrate has been studied extensively (Stewart, 1993). In contrast, little is known about the effect of TMAO addition on the synthesis of anaerobic systems. In this study we examined the influence of the presence of TMAO in the growth medium on the expression of formate hydrogenlyase and formate-nitrate reductase enzymic components and genes. We present evidence that metabolism of TMAO is essential for the severe repression of the formate hydrogenlyase complex in lowering the internal concentration of the inducer formate.

METHODS

Bacterial strains and plasmids. *E. coli* K-12-derived strains and plasmids used in this study are listed in Table 1. Derivative HA58 carrying the *fdnG* mutation was obtained using a P1cml lysate grown on strain VJS1224 (*fdnG*::MudII1734) (Berg & Stewart, 1990) to transduce strain MC4100 according to Miller (1992).

Media and growth conditions. Cells were grown aerobically on Luria-Bertani (LB) liquid or solid medium (Miller, 1992). Antibiotics were used at the following final concentrations: ampicillin (100 µg ml⁻¹), chloramphenicol (20 µg ml⁻¹) or kanamycin (50 µg ml⁻¹). When required, TMAO and formate were added at 40 mM and 30 mM, respectively.

For enzyme assays, bacterial strains were grown anaerobically at 37 °C in Tryptone Yeast Extract Phosphate (TYEP) medium (g l⁻¹: tryptone, 10; yeast extract, 5; K₂HPO₄, 12; KH₂PO₄, 3) adjusted to pH 6.5 (Begg *et al.*, 1977), supplemented with sodium selenite (2 µM) and ammonium molybdate (2 µM). For the measurement of β-galactosidase and β-glucuronidase activities, bacteria were grown to mid-exponential phase in undisturbed 25 ml screw-cap tubes. Analysis of anaerobic metabolism enzymes was performed after growth of cells in 500 ml bottles filled almost to the top, inoculated with 2.5 ml of overnight cultures grown in the same medium and tightly stoppered to maintain anaerobiosis. Cell samples were periodically collected to monitor OD₆₀₀ using a Kontron Uvikon 930 spectrophotometer.

Enzyme assays. Cell were washed twice and resuspended in 50 mM sodium/potassium phosphate buffer, pH 6.8, as previously described (Wu & Mandrand-Berthelot, 1986).

FDH-H, HYD, FDH-N and NAR were measured spectrophotometrically as described previously (Wu & Mandrand-

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype*	Source or reference
<i>E. coli</i>		
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>) <i>U169 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301</i>	Casadaban & Cohen (1979)
HA51	MC4100, <i>fdoG::uidA</i> (Km ^R)	Abaibou <i>et al.</i> (1995)
HA58	MC4100, <i>fdnG::MudII1734</i> (Km ^R <i>lac</i>)	This work
FD71	MC4100, <i>fdhF::MudI</i> (Ap ^R <i>lac</i>)	Wu & Mandrand-Berthelot (1987)
M17s	MC4100, <i>hycB::MudI</i> (Ap ^R <i>lac</i>)	Pecher <i>et al.</i> (1983)
LCB79	MC4100, ϕ 79(<i>narG::lacZ</i>)	Pascal <i>et al.</i> (1982)
LCB620	MC4100, <i>torA::MudII1734</i> (Km ^R <i>lac</i>)	Silvestro <i>et al.</i> (1989)
CS20	MC4100, <i>fdhD20::MudI</i> (Ap ^R <i>lac</i>)	This laboratory
CS6	MC4100, <i>fdhE6::MudI</i> (Ap ^R <i>lac</i>)	This laboratory
Plasmids		
pCS5	pACYC184 Cm ^R <i>fdhD</i> ⁺	Schlindwein <i>et al.</i> (1990)
pCS16	pACYC184 Cm ^R <i>fdhE</i> ⁺	Schlindwein <i>et al.</i> (1990)

* Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin.

Berthelot, 1986). TOR was measured by the same technique as NAR, but nitrate was replaced by 10 mM TMAO. Enzyme activities are expressed as μmol formate oxidized min^{-1} (mg bacterial dry wt)⁻¹ for FDH-H and FDH-N, as μmol benzyl viologen reduced min^{-1} (mg bacterial dry wt)⁻¹ for HYD, as μmol nitrate reduced min^{-1} (mg bacterial dry wt)⁻¹ for NAR, and as μmol TMAO reduced min^{-1} (mg bacterial dry wt)⁻¹ for TOR. Values are means of at least five independent experiments.

β -Galactosidase and β -glucuronidase activities were assayed in chloroform/SDS-permeabilized cells, according to the procedure described by Miller (1992).

RESULTS AND DISCUSSION

Effect of TMAO on the activity of anaerobic formate metabolism enzymes

The effect of supplementing anaerobic growth medium with TMAO was examined on the formate-degrading pathways in wild-type strain MC4100. The activity of the first component of the formate hydrogenlyase pathway, FDH-H, was totally abolished and the overall HYD activity was reduced 25% compared with levels observed without TMAO (Table 2). NAR activity was also decreased by more than twofold. In contrast, formate-dependent phenazine methosulphate reductase (FDH-PMS) activity, which consists of both FDH-N and FDH-Z isoenzymes, was stimulated by a factor of two. As expected, addition of TMAO, an efficient inducer of TOR (Silvestro *et al.*, 1989), led to a strong enhancement of TOR activity, which mainly reflects the presence of the inducible TOR in addition to the constitutive DMSO reductase (Weiner *et al.*, 1988). Furthermore, a direct effect of trimethylamine (TMA), the reduction product of TMAO, was discarded since it was shown not to affect the basal levels of the different enzyme activities.

Effect of TMAO on the expression of anaerobically related structural genes

To determine if the effect of TMAO is exerted at the transcriptional level on the structural genes corresponding to the above enzymes, we analysed the expression of *lacZ* or *uidA* operon fusions in the relevant genes in the presence of TMAO. As a control, expression of *torA*, the structural gene of the inducible TOR, was strictly dependent on the presence of its inducer TMAO under anaerobic conditions in agreement with a previous report (Pascal *et al.*, 1984) (Table 3). β -Galactosidase activities expressed from a *fdhF::lacZ* fusion (FD71, Wu & Mandrand-Berthelot, 1987) and a *hycB::lacZ* fusion (M17s, Rossmann *et al.*, 1991) were reduced sevenfold and eightfold, respectively. Therefore, transcription of the *fdhF* gene (encoding FDH-H) and the *hyc* operon (encoding HYD-3) is severely repressed by TMAO, which corroborates the previously observed suppression of FDH-H activity and a significant reduction in HYD activity.

HYD3 has been previously demonstrated to be the predominant HYD isoenzyme synthesized under fermentative conditions, accounting for at least 90% of the overall activity, whereas HYD1 and HYD2 are produced only in minor amounts (Sauter *et al.*, 1992). The persistence of a high level of HYD activity in cells grown in the presence of TMAO is likely to result from the induction of one or both of HYD1 and HYD2, which may 'compensate' for the loss of HYD3. In favour of this hypothesis a *lacZ* fusion in the *hya* operon encoding HYD1 was shown to be stimulated threefold by TMAO (data not shown). Induction by TMAO was considered to result from an indirect effect as no typical boxes for the binding of the regulatory protein TorR (Simon *et al.*, 1994) were found upstream of the *hya* operon. This

Table 2. Effect of TMAO on anaerobic enzyme activities in various strains

Bacteria were grown anaerobically at 37 °C in TYEP medium supplemented with 2 µM selenite and 2 µM molybdate and harvested at the end of the exponential growth phase. When required, TMAO and TMA were added at 40 mM. The results are the means of at least five independent experiments in which values did not vary by more than 15% of the mean.

Strain	Plasmid	Relevant genotype	Addition	Enzyme activity [units (mg bacterial dry wt) ⁻¹]				
				FDH-H	HYD	FDH-PMS	NAR	TOR
MC4100	–	Wild-type	None	0.12	1.20	0.13	0.48	0.30
			TMAO	0.005	0.90	0.25	0.18	1.10
			TMA	0.11	1.21	0.12	0.46	0.30
LCB620	–	<i>torA</i>	None	0.12	1.14	0.12	0.46	0.25
			TMAO	0.23	1.95	0.13	0.44	0.27
CS20	–	<i>fdhD</i>	None	0.12	1.18	0.002	0.46	0.28
			TMAO	0.07	1.00	0.005	0.30	1.12
	pCS5	<i>fdhD/fdhD</i> ⁺	None	0.12	1.20	0.11	0.48	0.30
			TMAO	0.006	0.88	0.24	0.20	1.11
CS6	–	<i>fdhE</i>	None	0.12	1.20	< 0.001	0.48	0.28
			TMAO	0.10	1.11	< 0.001	0.42	1.10
	pCS16	<i>fdhE/fdhE</i> ⁺	None	0.12	1.19	0.12	0.47	0.26
			TMAO	0.005	0.85	0.23	0.18	1.10
HA51	–	<i>fdoG</i>	None	0.11	1.16	0.11	0.46	0.28
			TMAO	0.006	0.88	0.23	0.19	1.11
HA58	–	<i>fdnG</i>	None	0.12	1.22	0.05	0.47	0.30
			TMAO	0.10	1.18	0.11	0.45	1.13

Table 3. Effect of TMAO and formate on *lacZ* and *uidA* operon fusions with various anaerobic enzyme genes

Strains were grown anaerobically at 37 °C in TYEP medium containing 2 µM selenite and 2 µM molybdate. When required, TMAO and formate were added at 40 mM and 30 mM, respectively. The results are the means of five separate determinations in which values did not deviate by more than 15% of the mean. ND, Not determined.

Strain	Relevant genotype	Specific activity of the fusion [units (mg bacterial dry wt) ⁻¹]			
		No addition	TMAO	Formate	TMAO + Formate
FD71	<i>fdhF::lacZ</i>	130	19	263	250
M17s	<i>hycB::lacZ</i>	120	15	244	231
HA58	<i>fdnG::lacZ</i>	82	87	ND	ND
HA51	<i>fdoG::uidA</i>	30	65	ND	ND
LCB79	<i>narG::lacZ</i>	175	184	ND	ND
LCB620	<i>torA::lacZ</i>	< 0.1	77	ND	ND

observation suggests a possible electron transfer from H₂ to TMAO via HYD1. A high level of hydrogen uptake in the presence of TMAO was also found by Yamamoto & Ishimoto (1978) who reported that growth of *E. coli* on peptone and TMAO was greatly stimulated by hydrogen.

On the other hand, β-galactosidase activities from both a *fdnG::lacZ* fusion (HA58) and a *narG::lacZ* fusion (LCB79, Pascal *et al.*, 1982) remained unchanged when

TMAO was present in the growth medium. This indicated that expression of the operons governing the synthesis of the two components of the major anaerobic formate-to-nitrate respiratory chain, FDH-N and NAR-A, is not affected by TMAO. We already noticed that FDH-PMS activity was stimulated in the presence of TMAO (Table 2). The discrepancy observed in gene expression may be partly accounted for by the existence of a second formate-dependent nitrate reduction pathway whose first component is FDH-Z (Pommier *et al.*,

1992; Abaibou *et al.*, 1995). In agreement with this suggestion, expression of the *fdoG::uidA* fusion reflecting the synthesis of the FDH-Z isoenzyme was increased 2.5-fold in the presence of TMAO (Table 3). It was not possible to explain the reduction of global NAR activity since the contribution of NAR-Z has been previously demonstrated to be very low with respect to the major anaerobic enzyme NAR-A (Iobbi-Nivol *et al.*, 1987). The absence of a regulatory control over the nitrate-inducible formate-to-nitrate respiratory chain was not surprising since nitrate is the preferred electron acceptor for anaerobic cells due to the more positive mid-point potential of the nitrate/nitrite couple (+430 mV) than that of the TMAO/TMA couple (+130 mV). It should also be noted that, unexpectedly, expression of TOR escapes the hierarchical repressive effect of nitrate (Pascal *et al.*, 1984). It has been previously established that expression of the *frd* and *dms* operons encoding fumarate reductase and DMSO reductase, respectively, is unaltered in the presence of TMAO (Jones & Gunsalus, 1987; Cotter & Gunsalus, 1989). Altogether these data indicate that the effect of regulation by TMAO is confined to the fermentative pathway.

Repression by TMAO results from anaerobic metabolism of formate to TMAO

The involvement of TMAO in the repression process was further investigated by measuring enzyme activities in a set of mutants impaired in the metabolism of TMAO. In the *torA* mutant LCB620 values identical to those of the wild-type strain MC4100 were obtained in the absence of TMAO (Table 2). However, when TMAO was supplied, not only were FDH-H and HYD activities not repressed, in contrast to the situation reported in the wild-type strain, but they were enhanced twofold over the basal levels found without TMAO. Similarly, we have previously observed that a series of mutations, abolishing TOR activity and located either in the *mol* (*chl*) genes required for molybdenum incorporation or in the *fnr* gene specifying the global activator of anaerobic respiration, led to the restoration of expression of gene *fdhF*, which specifies FDH-H, at the level found in the absence of TMAO (Wu & Mandrand-Berthelot, 1987).

Moreover, in the *fdhD* and *fdhE* mutants, CS20 and CS6, lacking both FDH-N and FDH-Z activities, levels of FDH-H and HYD were only slightly reduced by the presence of TMAO in the growth medium. Nevertheless, a slight repression of the components of the formate hydrogenlyase system was particularly obvious in the *fdhD* mutant whose weak residual FDH-PMS activity was induced 2.5-fold in the presence of TMAO. As expected, complementation of the *fdhD* and *fdhE* mutations by plasmids pCS5 and pCS16 carrying the wild-type alleles corresponding restored the repression pattern to the level observed in the wild-type strain MC4100 (Table 2).

The intracellular concentration of formate is known to

act as a unique signal for the induction of the formate hydrogenlyase genes (Rossmann *et al.*, 1991). An explanation for the low level or absence of repression of the formate hydrogenlyase pathway by TMAO in *fdhD*, *fdhE* or *torA* mutants would result from a level of formate sufficient for induction of the *fdhF* and *hyc* genes in these strains. In contrast, the presence of an alternative route for metabolism of formate to TMAO will reduce the amount of formate available for the induction of the formate hydrogenlyase system in the wild-type strain.

Relief of TMAO repression by formate

To test this hypothesis, the effect of supplementation of the medium with 30 mM formate on the expression of *fdhF::lacZ* and *hycB::lacZ* fusions carried by strains FD71 and M17s, respectively, grown anaerobically with 40 mM TMAO was examined. As can be seen in Table 3, addition of formate not only relieved the repression exerted by TMAO, but also stimulated transcription of *fdhF* and *hycB* to a level identical to that observed in the presence of formate alone, which led to a twofold enhancement with respect to the basal level. Accordingly, FDH-H and HYD activities were reliably elevated compared to normal basal levels in the wild-type strain grown in a medium containing formate in addition to TMAO (data not shown). Absence of formate metabolism in the *torA* mutant should lead to an excess of this compound which might explain the twofold stimulation of FDH-H and HYD activities in the presence of TMAO (Table 2). It is unknown why mutations of the two *fdhD* and *fdhE* genes do not fully restore expression of the formate hydrogenlyase genes (Table 2) since formate should concentrate upstream of the impaired FDH-PMS isoenzymes. A side electron flux from formate to TOR may be involved. FDH-H has been shown to contribute equally with FDH-N to the transfer of electrons from formate to nitrite (Darwin *et al.*, 1993). Its implication in the transfer of electrons to TOR cannot be excluded.

Altogether, these results are in agreement with the view that prevention of the formation of the formate hydrogenlyase pathway by TMAO is due to a limiting internal concentration of formate. Such an explanation has already been put forward for nitrate inhibition of formate hydrogenlyase formation by Böck's group who found that the repressive effect of nitrate could be partially relieved either by adding exogenous formate or by introducing a mutation in the NAR genes (Rossmann *et al.*, 1991). In contrast, it should be mentioned that the two alternative anaerobic electron acceptors tested, fumarate and DMSO, had no effect on formate hydrogenlyase expression (Wu & Mandrand-Berthelot, 1987; Pecher *et al.*, 1983).

Growth of mutants impaired in FDH-PMS and TOR activities in the presence of TMAO

Since all these results clearly show that oxidation of formate by TMAO is responsible for the repressive effect of TMAO on the expression of the fermentative

Fig. 1. Effect of TMAO on the anaerobic growth of strains impaired in formate dehydrogenase and TOR activities. Cells were grown anaerobically at 37 °C in 500 ml bottles filled almost to the top with TYEP medium supplemented with 2 μ M selenite and 2 μ M molybdate in the absence (a) or presence (b) of 40 mM TMAO. Growth was estimated by measurement of OD₆₀₀. ●, Wild-type MC4100; △, *fdoG* mutant HA51; ○, *fdnG* mutant HA58; ▲, *torA* mutant LCB620. The experiments were carried out at least twice. The results reported are from a representative experiment.

formate hydrogenlyase pathway, we wanted to assess the role of the two FDH-N and FDH-Z isoenzymes in the formate-TMAO respiratory pathway. To this end, the *fdoG* mutant HA51 and the *fdnG* mutant HA58 were grown anaerobically in the absence or presence of TMAO and enzyme activities were measured. Strains MC4100 (wild-type) and LCB620 (*torA*) were included for comparison. Whereas all four strains exhibited identical growth in TYEP medium, they could be separated into two classes with respect to their behaviour in the presence of TMAO (Fig. 1). Growth of strains MC4100 and HA51 was greatly stimulated by addition of 40 mM TMAO in the medium in contrast to that of strains HA58 and LCB620 which remained unchanged. It thus appears that FDH-N and TOR activities, both of which are present in the wild-type strain MC4100 and in the *fdoG* mutant HA51, are required for the *in vivo* transfer of electrons from formate to TMAO under anaerobic conditions.

These results were further substantiated by analysis of enzyme activities in the *fdoG* and *fdnG* mutants. In the

fdoG mutant HA51, which possesses the sole FDH-N isoenzyme, levels of FDH-H and HYD activities were repressed to a similar rate as that observed in the parental strain in the presence of TMAO (Table 2). In contrast, enzymes of the formate hydrogenlyase pathway were barely affected by addition of TMAO in the *fdnG* mutant HA58 harbouring only the FDH-Z isoenzyme. It should also be noted that global FDH-PMS activity was stimulated by a factor of two in all strains tested except in the *torA* mutant LCB620 (Table 2). An excess of formate in this strain might explain this phenotype, since addition of formate has already been shown to lower the synthesis of FDH-PMS activity (Schlindwein *et al.*, 1990) and thus it may antagonize the inductive effect of TMAO.

This study demonstrates that FDH-N can participate in respiration with TMAO in addition to its function with nitrate and nitrite. In contrast, the constitutive DMSO reductase present in all cases seems to play a relatively minor role in draining the electron flux from formate. This is clear from the fact that in the *torA* mutant which displays DMSO reductase activity, not only was the formate hydrogenlyase system no longer inhibited by TMAO, but also no growth enhancement was visible with TMAO (Fig. 1). In agreement with the minor contribution of DMSO reductase to the *in vivo* reduction of TMAO is the observation of Daruwala & Meganathan (1991) who found a similar growth rate and cell yield of the *dms* mutants and the wild-type strain after growth on glycerol supplemented with TMAO. However, studies with *torA* mutants have also shown that the DMSO reductase is sufficient to sustain growth on TMAO (Sambasivarao & Weiner, 1991).

Interestingly, enhancement by TMAO of FDH-Z synthesis, resulting from the elevated transcription of its relevant *fdo* structural operon (Tables 2 and 3), suggests that this enzyme might have a function in the transfer of electrons to TOR and substitute for a loss of FDH-N isoenzyme. However, since no repression of formate hydrogenlyase expression was observed in a *fdnG* mutant in which FDH-Z is expressed, formate oxidation via FDH-Z and TOR is likely to proceed with a low efficiency.

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REFERENCES

- Abaibou, H., Pommier, J., Benoit, S., Giordano, G. & Mandrand-Berthelot, M. A. (1995). Expression and characterization of the *Escherichia coli fdo* locus and a possible physiological role for aerobic formate dehydrogenase. *J Bacteriol* 177, 7141-7149.

- Barrett, E. L. & Kwan, H. S. (1985). Bacterial reduction of trimethylamine oxide. *Annu Rev Microbiol* **39**, 131–149.
- Begg, Y. A., Whyte, J. N. & Haddock, B. A. (1977). The identification of mutants of *Escherichia coli* deficient in formate dehydrogenase and nitrate reductase activities using dye indicator plates. *FEMS Microbiol Lett* **2**, 47–50.
- Berg, B. L. & Stewart, V. (1990). Structural genes for nitrate-inducible formate dehydrogenase in *Escherichia coli* K-12. *Genetics* **125**, 691–702.
- Berg, B. L., Li, J., Heide, J. & Stewart, V. (1991). Nitrate-inducible formate dehydrogenase in *Escherichia coli* K-12: I. Nucleotide sequence of the *fdnGHJ* operon and evidence that opal (UGA) encodes selenocysteine. *J Biol Chem* **266**, 22380–22385.
- Bilous, P. T. & Weiner, J. H. (1988). Molecular cloning and expression of the *Escherichia coli* dimethyl sulfoxide reductase operon. *J Bacteriol* **170**, 1511–1518.
- Blasco, F., Iobbi, C., Giordano, G., Chippaux, M. & Bonnefoy, V. (1989). Nitrate reductase of *Escherichia coli*: completion of the nucleotide sequence of the *nar* operon and reassessment of the role of the α and β subunits in iron binding and electron transfer. *Mol Gen Genet* **218**, 249–256.
- Blasco, F., Iobbi, C., Ratouchniak, J., Bonnefoy, V. & Chippaux, M. (1990). Nitrate reductases of *Escherichia coli*: sequence of the second nitrate reductase and comparison with that encoded by *narGHJ* operon. *Mol Gen Genet* **222**, 104–111.
- Böck, A. & Sawers, G. (1996). Fermentation. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 262–282. Edited by F. C. Neidhardt. Washington, DC: American Society for Microbiology.
- Casadaban, M. & Cohen, S. N. (1979). Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: *in vivo* probe for transcriptional control sequences. *Proc Natl Acad Sci USA* **76**, 4530–4533.
- Cotter, P. A. & Gunsalus, R. P. (1989). Oxygen, nitrate, and molybdenum regulation of *dmsABC* gene expression in *Escherichia coli*. *J Bacteriol* **171**, 3817–3823.
- Darwin, A., Tormay, P., Page, L., Griffiths, L. & Cole, J. (1993). Identification of the formate dehydrogenases and genetic determinants of formate-dependent nitrite reduction by *Escherichia coli* K12. *J Gen Microbiol* **139**, 1829–1840.
- Daruwala, R. & Meganathan, R. (1991). Dimethyl sulfoxide reductase is not required for trimethylamine N-oxide reduction in *Escherichia coli*. *FEMS Microbiol Lett* **83**, 255–260.
- Gennis, R. B. & Stewart, V. (1996). Respiration. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 217–261. Edited by F. C. Neidhardt. Washington, DC: American Society for Microbiology.
- Iobbi-Nivol, C., Santini, C. L., Bonnefoy, V. & Giordano, G. (1987). Biochemical and immunological evidence for a second nitrate reductase in *Escherichia coli* K-12. *Eur J Biochem* **168**, 451–459.
- Iobbi-Nivol, C., Santini, C. L., Blasco, F. & Giordano, G. (1990). Purification and further characterization of the second nitrate reductase of *Escherichia coli* K-12. *Eur J Biochem* **188**, 679–687.
- Jones, H. M. & Gunsalus, R. P. (1987). Regulation of *Escherichia coli* fumarate reductase (*frdABCD*) operon expression by respiratory electron acceptors and the *fnr* gene product. *J Bacteriol* **169**, 3340–3349.
- Jourlin, C., Bengrine, A., Chippaux, M. & Méjean, V. (1996a). An unorthodox sensor protein (TorS) mediates the induction of the *tor* structural genes in response to trimethylamine N-oxide in *Escherichia coli*. *Mol Microbiol* **20**, 1297–1306.
- Jourlin, C., Simon, G., Pommier, J., Chippaux, M. & Méjean, V. (1996b). The periplasmic TorT protein is required for trimethylamine-N-oxide reductase gene induction in *Escherichia coli*. *J Bacteriol* **178**, 1219–1223.
- Miller, J. H. (1992). Use of P1clr100. In *A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*, pp. 275–278. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Pascal, M. C., Burini, J. F., Ratouchniak, J. & Chippaux, M. (1982). Regulation of the nitrate reductase operon: effect of mutations in *chlA*, *B*, *D* and *E* genes. *Mol Gen Genet* **188**, 103–106.
- Pascal, M. C., Burini, J. F. & Chippaux, M. (1984). Regulation of trimethylamine N-oxide (TMAO) reductase of *Escherichia coli* K-12: analysis of *tor::MudI* fusion. *Mol Gen Genet* **195**, 351–355.
- Pecher, A., Zinoni, F., Jatisatienr, C., Wirth, R., Hennecke, H. & Böck, A. (1983). On the redox control of synthesis of anaerobically induced enzymes in enterobacteriaceae. *Arch Microbiol* **136**, 131–136.
- Plunkett, G., Burland, V., Daniels, D. L. & Blattner, F. R. (1993). Analysis of the *Escherichia coli* genome. III. DNA sequence of the region from 87.2 to 89.2 minutes. *Nucleic Acids Res* **21**, 3391–3398.
- Pommier, J., Mandrand-Berthelot, M. A., Holt, S. E., Boxer, D. H. & Giordano, G. (1992). A second phenazine methosulphate-linked formate dehydrogenase isoenzyme in *Escherichia coli*. *Biochim Biophys Acta* **1107**, 305–313.
- Rossmann, R., Sawers, G. & Böck, A. (1991). Mechanism of regulation of the formate-hydrogenlyase pathway by oxygen, nitrate, and pH: definition of the formate regulon. *Mol Microbiol* **5**, 2807–2814.
- Rossmann, R., Maier, T., Lottspeich, F. & Böck, A. (1995). Characterisation of a protease from *Escherichia coli* involved in hydrogenase maturation. *Eur J Biochem* **227**, 545–550.
- Sambasivarao, D. & Weiner, J. H. (1991). Differentiation of the multiple S- and N-oxide-reducing activities of *Escherichia coli*. *Curr Microbiol* **23**, 105–110.
- Sauter, M., Böhm, R. & Böck, A. (1992). Mutational analysis of the operon (*hyc*) determining hydrogenase 3 formation in *Escherichia coli*. *Mol Microbiol* **6**, 1523–1532.
- Schindwein, C., Giordano, G., Santini, C. L. & Mandrand, M. A. (1990). Identification and expression of the *Escherichia coli* *fdhD* and *fdhE* genes, which are involved in the formation of respiratory formate dehydrogenase. *J Bacteriol* **172**, 6112–6121.
- Silvestro, A., Pommier, J., Pascal, M. C. & Giordano, G. (1989). The inducible trimethylamine N-oxide reductase of *Escherichia coli* K-12: its localization and inducers. *Biochim Biophys Acta* **994**, 208–216.
- Simon, G., Méjean, V., Jourlin, C., Chippaux, M. & Pascal, M. C. (1994). The *torR* gene of *Escherichia coli* encodes a response regulator protein involved in the expression of the trimethylamine-N-oxide reductase genes. *J Bacteriol* **176**, 5601–5606.
- Sodergren, E. J. & DeMoss, J. A. (1988). *narI* region of nitrate reductase (*nar*) operon contains two genes in *Escherichia coli*. *J Bacteriol* **170**, 1721–1729.
- Stewart, V. (1993). Nitrate regulation of anaerobic respiratory gene expression in *Escherichia coli*. *Mol Microbiol* **9**, 425–434.
- Stewart, V., Lin, J. T. & Berg, B. L. (1991). Genetic evidence that genes *fdhD* and *fdhE* do not control synthesis of formate dehydrogenase-N in *Escherichia coli* K-12. *J Bacteriol* **173**, 4417–4423.
- Weiner, J. H., Macisaac, D. P., Bishop, R. E. & Bilous, P. T. (1988). Purification and properties of *Escherichia coli* DMSO reductase,

an iron-sulfur molybdoenzyme with broad substrate specificity. *J Bacteriol* **170**, 1505–1510.

Wu, L. F. & Mandrand-Berthelot, M. A. (1986). Genetic and physiological characterization of new *Escherichia coli* mutants impaired in hydrogenase activity. *Biochimie* **68**, 167–179.

Wu, L. F. & Mandrand-Berthelot, M. A. (1987). Regulation of the *fdhF* gene encoding the selenopolypeptide for benzyl viologen-linked formate dehydrogenase in *Escherichia coli*. *Mol Gen Genet* **209**, 129–134.

Yamamoto, I. & Ishimoto, M. (1978). Hydrogen-dependent growth

of *Escherichia coli* in anaerobic respiration and the presence of hydrogenases with different functions. *J Biochem* **84**, 673–679.

Zinoni, F., Birkmann, A., Stadman, T. C. & Böck, A. (1986). Nucleotide sequence and expression of selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogen-lyase-linked) from *Escherichia coli*. *Proc Natl Acad Sci USA* **83**, 4650–4654.

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