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A second phenazine methosulphate-linked formate dehydrogenase isoenzyme in *Escherichia coli*

Janine Pommier [a], Marie André Mandrand [b], Susan E. Holt [c], David H. Boxer [c] and Gérard Giordano [a]

[a] *Laboratoire de Chimie Bacterienne, CNRS, Marseille (France)* [b] *Department of Microbiology, INSA, Villeurbonne (France) and* [c] *Department of Biochemistry, Medical Sciences Institute, Dundee University, Dundee Scotland (UK)*

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A biochemical and immunological study has revealed a new formate dehydrogenase isoenzyme in *Escherichia coli*. The enzyme is an isoenzyme of the respiratory formate dehydrogenase (FDH-N) which forms part of the formate to nitrate respiratory pathway found in the organisms when it is grown anaerobically in the presence of nitrate. The new enzyme, termed FDH-Z, cross reacts with antibodies raised to FDH-N and possesses a similar polypeptide composition to FDH-N. FDH-Z catalyses the phenazine methosulphate-linked formate dehydrogenase activity present in the aerobically-grown bacterium. FDH-Z and FDH-N exhibit distinct regulation. Like formate dehydrogenase N, formate dehydrogenase Z is a membrane-bound molybdoenzyme. With nitrate reductase it can catalyse electron transfer between formate and nitrate. Quinones are required for the physiological electron transfer to nitrate. It seems likely that like FDH-N, FDH-Z functions physiologically as a formate : quinone oxidoreductase.

Introduction

Escherichia coli has two pathways for the anaerobic metabolism of formate. The respiratory pathway, formate-dependent nitrate reduction is responsible for reduction of nitrate to nitrite via quinones and cytochromes *b* [1,2]. This pathway involves two membrane-bound multisubunit enzymes, formate dehydrogenase N (FDH-N) and nitrate reductase. Both enzymes are induced by nitrate during anaerobic growth. The respiratory pathway catalyzes the formate-dependent reduction of nitrate to nitrite coupled to proton translocation with the generation of ATP by oxidative phosphorylation [3]. Purified formate dehydrogenase N consists of three subunits (α, β and γ) of relative molecular mass 110000, 32000 and 20000, respectivcly. The α-subunit contains selenocysteine, probably binds the molybdenum cofactor and contains the site of formate oxidation. The α- and β-subunits occupy transmembranous positions in the cytoplasmic membrane and the heme is thought to be associated with the γ-subunit [4,5]. FDH-N has been characterized as a formate–phenazinemethosulfate oxidoreductase.

The three subunits of FDH-N are encoded by the *fdn* GHI operon at 32 min on the *E. coli* genetic map. Expression of a ϕ(*fdnG'-lacZ*) operon fusion was induced by anaerobiosis and nitrate. This induction required the functional products of *fnr* and *narL*, two regulatory genes which are also required for the anaerobic nitrate-dependent induction of the nitrate reductase structural operon, *narGHJI* [6].

The second pathway of formate metabolism, formate-hydrogen lyase, operates anaerobically in the absence of nitrate, decomposing formate to hydrogen and carbon dioxide [7,8]. This pathway involves two enzymes, formate dehydrogenase H (FDH-H) and an hydrogenase [3]. The expression of FDH-H is elevated by formate but repressed by oxygen, nitrate, nitrite and other respiratory electron acceptors. Formate can overcome the repression by nitrate but not by oxygen [9,10]. The *fdhF* gene, which encodes the FDH-H 90 kDa polypeptide, is located at 92.4 min on the chromosomal map. Transcription of FDH-H requires both NTRA, an alternative sigma factor which directs RNA polymerase to specific promoters. The FNR protein, a

Correspondence to: G. Giordano, Laboratoire de Chimie Bacterienne, CNRS, BP71, 31 Chemin Joseph Aiguier, 13277 Marseille Cedex 09, France.

Abbreviation: FDH-N, phenazine methosulphate-linked formate dehydrogenase.

Enzymes: formate dehydrogenase (EC 1.2.2.1); nitrate reductase (EC 1.7.99.4).

positive transcriptional regulator of many anaerobic respiratory genes is not required for FDH-H expression [11–14]. Active FDH-H has been purified recently, characterised as a benzyl viologen oxidoreductase and as formate dehydrogenase N, is a selenoprotein [15].

Two nitrate reductases, nitrate reductase A and nitrate reductase Z, exist in *E. coli* [16,17]. Both enzymes are composed of three subunits α, β and γ, encoded by the *narG/narZ*, *narH/narY* and *narI/narV* genes, respectively. Nucleotide sequence analysis shows that the structural genes of both enzymes are very similar and are organized in a similar manner [18,19]. The *narZYWV* and *narGHJI* operons have probably descended from a common ancestor [18,19]. The two nitrate reductases are very similar with respect to relative molecular mass, subunit composition and specificity for electron donors and acceptors. An immunological study demonstrated epitopes common to both enzymes. The regulation of both enzymes is, however, distinct [16,20].

Recently Sawers et al. [21] have discovered that *E. coli* possesses a third selenoprotein, different from formate dehydrogenase H and formate dehydrogenase N. Its expression is similar during both aerobic and anaerobic growth with nitrate. In their work several points argue in favour of a 110 kDa protein being a subunit of a third formate dehydrogenase isoenzyme.

In this report we describe immunological experiments which reveal a third formate dehydrogenase isoenzyme in *E. coli*. The enzyme appears to be closely related to formate dehydrogenase N.

Materials and Methods

Bacterial strains. The *Escherichia coli* strains used and their genotypes are listed in Table I. The *narG* and *narZ* mutations were introduced into the strains of interest, by bacteriophage P1-mediated transduction using the kanamycin or spectinomycin resistance markers. Plasmid pLCB14 (18.9 kb) is a pBR322 derivative carrying the *narZYWV* operon [17].

Media and growth conditions. The strains were grown in L-broth medium supplemented with glucose (0.2%, w/v), sodium selenite (2 μM) and ammonium molybdate (2 μM) [26]. When indicated, potassium nitrate (1%, w/v) or sodium tungstate (10 mM) were added. Anaerobic growth was accomplished at 37°C in non-agitated closed vessels filled almost to the top with medium. Strains carrying plasmid pLCB14 were grown in media containing ampicillin (50 μg/ml).

Preparation of subcellular fractions. The cells were harvested during the exponential phase of growth, suspended in 50 mM Tris-HCl, 1 mM benzamidine-HCl (pH 7.6) and ruptured in a French Press. The crude extract was centrifuged at 18000 × *g* for 15 min. The supernatant fraction was further centrifuged at 170000 × *g* for 90 min, and the soluble and membrane fractions recovered. All procedures were performed at 4°C.

TABLE I

E. coli strains used in this work

Strain	Genotype	Reference
MC4100	*araD139Δ (lacIPOZYA-argF) rpsL, thi*	22
LCB79	MC4100 with ϕ79 (*nar-lac*)	17
LCB320	*thi-1, thr-1, leu-6, lacY1*, Sup E44, *rpsL 175*	23
LCB333	LCB320 with *Δnar25* (*nar G-H'*, Km^r)	23
LCB2048	LCB333 with *narZ*::Ω (spc^r)	23
RK5278	MC4100 with *gyrA 219 non9 narL 215::Tn10*	6
LCB22	*thr1, leu6, lacY1, rpsL175, ana1, fnr* (*nirR22*)	20
Fd17	*fdhD1*	25
AN387	*thi*, str^r	24
AN384	AN387 with *ubiA 420 men A401*	24
AN385	AN387 with *ubiA 420*	24
AN386	AN387 with *menA 401*	24
$AN385_1$	AN385 with *Δnar25* (*narG-H'*, Km^r), *narZ*::Ω (spc^r)	this work
$AN386_1$	AN386 with *Δnar25* (*narG-H'*, Km^r) *narZ*::Ω (spc^r)	this work
$AN384_1$	AN384 with *Δnar25* (*narG-H'*, Km^r) *narZ*::Ω (spc^r)	this work

Enzyme assays. Nitrate reductase activity was measured spectrophotometrically at 30°C following at 600 nm, the oxidation of reduced benzyl viologen by nitrate [27]. One unit of nitrate reductase activity is that amount catalysing the production of 1 μmole nitrite/min.

Formate dehydrogenase activity was assayed spectrophotometrically at 30°C by monitoring the formate-dependent, phenazine methosulfate (PMS)-mediated reduction of 2,6-dichlorophenolindophenol (DCPIP) as described by Lester and DeMoss [28].

Formate-dependent nitrate reduction was measured in whole cells with formate added as electron donor [29]. The reaction was stopped by addition of acetone. After centrifugation, nitrite was determined in an aliquot of the supernatant by the method of Rider and Mellon [30].

The concentration of protein was determined by the technique of Lowry et al. [31].

Polyacrylamide gel electrophoresis. Non-denaturing electrophoresis was carried out in 7.5% (w/v) polyacrylamide gels at pH 8.8. Direct localization of activity was achieved by the method of Scott and DeMoss [32]. The two active bands obtained ($R_F = 0.22$, $R_F = 0.14$) were cut out from the native gels, electroeluted and loaded on a SDS-polyacrylamide gel [33] for analysis by the Western immunoblot method. SDS-polyacrylamide

gel electrophoresis was done as described by Laemmli [53].

Immunological analysis. Antiserum to purified *E. coli* phenazine methosulphate-linked formate dehydrogenase N [34] was raised in rabbits immunized with enzyme purified as described by Enoch and Lester [4].

Rocket immunoelectrophoresis. Detection of formate dehydrogenase antigen present in Triton X-100-dispersed membrane fractions was achieved by rocked immunoelectrophoretic analysis as described by Graham et al. [35]. The samples were electrophoresed at 2 mA overnight in 4 × 4 cm (1%, w/v) agarose (1%, w/v) plates buffered with 20 mM sodium barbital (pH 8.6) containing Triton X-100 and sodium azide (0.05%, w/v). Antiserum (180 μl) was included in the agarose medium.

Western immunoblot analysis. After electrophoresis in 7.5% (w/v) SDS-polyacrylamide gels, protein was electrotransferred to a nitrocellulose sheet in methanol 20% (w/v) containing buffer [36]. The blots were exposed for 90 min at 37°C to Regilait milk (5%, w/v) in 10 mM Tris-HCl, 150 mM NaCl, Tween 20 (pH 8, 0.05% w/v). The blots were then incubated for 1 h at room temperature with anti-formate dehydrogenase serum (40 μl/10 ml buffer). After several washes, the immunoblots were incubated with anti-IgG second antibodies conjugated with alkaline phosphatase (Protoblot Western Blot AP-Rabbit-Promega). Sites of antigen localization were revealed by staining for alkaline phosphatase activity.

Double immunodiffusion analysis. Double immunodiffusion analyses were performed in agar (1%, w/v) and Triton X-100 (0.05%, w/v) as described by Ouchterlony [37].

Results

Formate dehydrogenase activity in aerobically grown E. coli

Previous work has shown that phenazine methosulphate-linked formate dehydrogenase activity (FDH-N) is repressed by oxygen but induced by nitrate during anaerobic growth [33]. Crude extracts of aerobically grown *E. coli*, however, possess a significant amount of this activity (Table II). The level of the activity in crude extracts of aerobically grown cells is about a third of the fully induced anaerobic level, but is unaffected by the presence of nitrate in the growth medium.

In a recent report, Berg and Stewart [6] demonstrated that the structural operon encoding the anaerobic enzyme (FDH-N) is expressed under the positive control of two regulatory genes *fnr* and *narL*. Our data show that crude extracts of *fnr* and *narL* mutants display aerobic formate dehydrogenase activity at more or less the same level as that of the parental strain (Table II). Neither of these genes are therefore required for the observed aerobic expression of phenazine methosulphate-linked formate dehydrogenase activity.

TABLE II

Formate dehydrogenase activity in crude extracts of parental strain and fnr and narL mutants

Strains	Formate dehydrogenase activity [a]		
	anaerobiosis +KNO_3	aerobiosis	
		+KNO_3	−KNO_3
MC4100	0.52	0.17	0.15
MC4100 [b]	0.04	0.02	0.02
LCB22 (*fnr*)	0.15	0.18	0.19
RK5278 (*narL*::*Tn10*)	0.17	0.16	0.19
Fd17 (*fdhD1*)	< 0.001	< 0.001	< 0.001

[a] Assayed as phenazine methosulphate-linked formate dehydrogenase and expressed as μmole formate oxidized per min per mg of protein.

[b] In this case, growth medium contained 10 mM sodium tungstate.

Biochemical properties of aerobically-expressed formate dehydrogenase

(a) Subcellular localization. An analysis of the subcellular distribution of this formate dehydrogenase activity in cells grown under aerobic conditions revealed that more than 80% was found in the membrane fraction even after two consecutive ultracentrifugations of the crude extract at 170000 × *g*.

(b) Effect of sodium tungstate. When *E. coli* is grown in media containing sodium tungstate, a structural analogue of molybdate, the activity of its molybdoenzymes is very low [28,38]. Under these conditions, we obtained only 12% of normal phenazine methosulphate linked formate dehydrogenase activity in the crude extract of aerobically grown cells (Table II). These observations strongly suggest that a molybdoenzyme is responsible for the aerobically expressed activity.

(c) Non-denaturing polyacrylamide gel analysis. Triton X-100-dispersed membrane fractions were examined following electrophoresis in 7.5% (w/v) polyacrylamide gels for protein displaying formate dehydrogenase activity. Direct localization of activity (see Materials and Methods) of the membrane-bound fraction of cells grown anaerobically in the presence of nitrate showed two active bands $R_F = 0.14$ and $R_F = 0.22$. The active band of $R_F = 0.14$ appeared to possess far more activity than the band of $R_F = 0.22$. When cells were grown aerobically, only one active band was detected at $R_F = 0.22$. Furthermore, in mutant strain LCB22 (carrying a mutated allele of *fnr*), in which the anaerobic formate dehydrogenase-N (FDH-N) is not synthesized, the band of $R_F = 0.22$ was the only band present under all growth conditions examined (data not shown).

These results suggest that the enzyme responsible for the aerobic phenazine methosulphate-linked for-

mate dehydrogenase activity is distinct from the enzyme characterized as catalysing this activity in anaerobically grown cells (FDH-N). The aerobic enzyme exhibits an electrophoretic, R_F, of 0.22 while the anaerobic (FDH-N) enzyme migrates with an R_F of 0.14. We term the enzyme of R_F 0.22, formate dehydrogenase Z (FDH-Z).

Immunological study of formate dehydrogenase Z

We recently reported that *E. coli* grown under aerobic conditions where FDH-N is not synthesized, possesses a protein which cross-reacts with antibodies raised against the FDH-N enzyme. These results led us to speculate that this protein may be a second FDH-N isoenzyme [39]. In order to test this hypothesis, several complementary immunological approaches were performed using a serum raised against the purified FDH-N enzyme. Since the putative second FDH isoenzyme may be weakly recognized by the antiserum directed against FDH-N, six-fold higher concentrations than usual were used in the analysis [25,34]. The possibility that the preparation of FDH-N used to raise antibodies may have contained trace amounts of FDH-Z cannot be excluded.

(a) Rocket immunoelectrophoretic analysis. Rocket immunoelectrophoresis of Triton X-100-dispersed membrane fractions of cells grown anaerobically with nitrate revealed two precipitin arcs (Fig. 1) one of which (arc 2) was not visible in our previous experiments which employed lower serum concentrations [25,34]. This second precipitin arc (stained with Coomassie blue) was less intense and far larger than arc 1 (Fig. 1). Nevertheless, the height of both arcs was proportional to the amount of total protein applied.

A similar analysis, performed with the membrane fraction of cells grown aerobically in the presence or in the absence of nitrate, revealed a single large precipitin arc of low intensity. Examination of Fig. 1 shows that formate dehydrogenase Z is also synthesized under anaerobic conditions since, when tested in equivalent amounts of total protein, the anaerobically expressed arc 2 was about the same size as the aerobic arc.

We were able to detect immunoprecipitin arcs with a phenazine methosulfate-linked enzyme stain, prior to staining for protein with Coomassie blue (data not shown). This zymogram stain eliminates the possibility that the second arc may be a non-specific protein band. Taken together, these results reveal that *E. coli* contains two different phenazine methosulfate-linked formate dehydrogenase enzymes, one of which is poorly recognized by the serum raised against FDH-N.

(b) Double immunodiffusion analysis. Triton X-100 membrane extracts of aerobically grown cells revealed only a single precipitin arc when protein amounts higher than 120 μg were used (Fig. 2, d, f). Under the same conditions the membrane fraction of cells grown anaerobically in the presence of nitrate gave a very intense large arc which did not allow a proper assessment of the presence of further precipitin arcs (data not shown). However, a single precipitin arc appeared when four times less protein (30 μg) was used (Fig. 2, a, b, e). The absence of a second arc was expected since no precipitin arc was detected in aerobically grown cells with the lower amount (30 μg) of protein. All precipitin arcs were enzymatically active.

The important feature of this experiment was that the immunoprecipitin arc found with anaerobically grown cells did not fuse completely with that obtained from aerobically grown cells. This can be seen in Fig. 2 (a, f) where a spur extending from immunoprecipitin arc from anaerobically grown cells can be clearly distin-

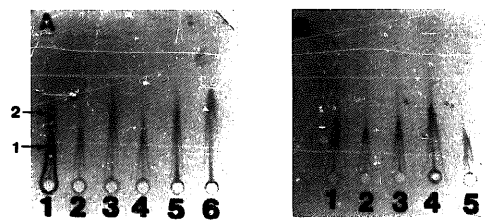

Fig. 1. Analysis by rocket immunoelectrophoresis of the phenazine methosulphate mediated formate dehydrogenase activity in various strains grown anaerobically and aerobically with nitrate. Rocket immunoelectrophoresis was performed as described in Materials and Methods. Each well received 6 μl membrane extract solubilized with Triton X-100 (60 μg protein). Each plate was poured with 3.3 ml 1% (w/v) agarose containing 180 μl anti-formate dehydrogenase N serum. (A) (1) strain MC4100 grown anaerobically with nitrate, (2) aerobically with nitrate and (3) aerobically, (4) mutant LCB22 (*fnr*) grown anaerobically with nitrate, (5) aerobically with nitrate and (6) aerobically. (B) (1) mutant RK5278 (*narL*::*Tn10*) grown aerobically, (2) aerobically with nitrate, (3) mutant FD17 (*fdhD*) grown aerobically, (4) aerobically with nitrate and (5) LCB2048 grown aerobically with nitrate. Arrows 1 and 2 indicate FDH-N and FDH-Z immunoprecipitates, respectively.

guished. These observations demonstrate that the anaerobically inducible enzyme FDH-N has common epitopes with the aerobic formate dehydrogenase Z.

These findings were confirmed when a membrane extract of an *fnr* from mutant (LCB22) which does not synthesize the FDH-N enzyme was analysed. High protein amounts (120 μg) were required for the detection of a single immunoprecipitin arc from the mutant whether grown aerobically or anaerobically with nitrate. This arc fused perfectly with that from the aerobically grown parental strain (MC4100) whether in the absence or presence of nitrate (Fig. 2, c, d). This indicates that formate dehydrogenase activity in the parental strain grown aerobically and in the *fnr* mutant, is due to formate dehydrogenase Z. As expected, a spur was formed between the precipitin arc of the anaerobically induced FDH-N from the parental strain and the formate dehydrogenase Z from the *fnr* strain (Fig. 2, b, c). Similar results were obtained for a *narL* mutant.

(c) Subunit composition of formate dehydrogenase Z. In order to further characterize the aerobically synthesized formate dehydrogenase Z, we determined its subunit composition and compared it with that of the anaerobically inducible FDH-N. Fig. 3 shows that formate dehydrogenase Z contains two subunits, α and β, having relative molecular masses around 100 000 and 32 000, respectively. These values are similar to those of the α and β subunits of FDH-N (110 000 and 32 000). The α-subunits of the isoenzymes are of clearly different mobilities but the β-subunits could not be distinguished. It is possible that the β-subunits of the enzymes are identical. Our analyses were of insufficient resolution to reveal the γ-subunit of FDH-N so

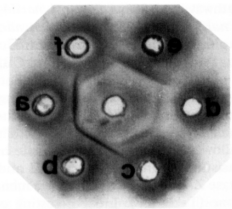

Fig. 2. Ouchterlony double immunodiffusion of solubilized membrane extracts of strains MC4100 and of mutant LCB22 (*fnr*). Triton X-100-solubilized membrane extracts of various strains and anti-formate dehydrogenase N serum (25 μl) was placed in wells as indicated below. Diffusion was allowed at room temperature for 48 h. Precipitin arcs were revealed by staining with Coomassie blue (a,b,e) strain MC4100 grown in anaerobiosis with nitrate (30 μg), (d,f) strain MC4100 grown in aerobiosis with nitrate (120 μg) and (c) mutant LCB22 (*fnr*) grown in aerobiosis with nitrate (120 μg).

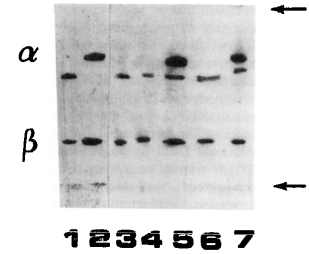

Fig. 3. Analysis by Western immunoblot of the subunit composition of formate dehydrogenase of parental strain and of mutant LCB22 (*fnr*) grown in anaerobiosis and aerobiosis with nitrate. Both active formate dehydrogenase bands ($R_F = 0.14$, $R_F = 0.22$), found in the non-denaturing polyacrylamide gel after electrophoresis of the solubilized membrane extract of strain MC4100, were cut out and the enzymes electroeluted. These samples were electrophoresed as were the solubilized membrane extracts (2 μg of protein) in SDS-polyacrylamide gels (7.5%, w/v) and immunoblotted as described in Materials and Methods. 1, Active band of $R_F = 0.22$ from solubilized membrane extract of strain MC4100 grown in anaerobiosis with nitrate. 2, Active band of $R_F = 0.14$ from solubilized membrane extract of strain MC4100 grown in anaerobiosis with nitrate. 3, Solubilized membrane extract of mutant LCB22 (*fnr*) grown in aerobiosis with nitrate. 4, Solubilized membrane extract of mutant LCB22 (*fnr*) grown in anaerobiosis with nitrate. 5 and 7, Solubilized membrane extract of parental strain MC4100 grown in anaerobiosis with nitrate. 6, Solubilized membrane extract of parental strain MC4100 grown aerobically. The arrows indicate the top of the gel and the dye front.

we are unable to assess the possible presence of a γ-subunit in FDH-Z. Similar difficulties in the detection of the γ-subunits of the nitrate reductases have been reported previously [16,20]. The subunit composition of the enzymes from the solubilized membrane extract strains MC4100 and LCB22 (*fnr*) are in agreement with our immunological and biochemical findings reported above (Fig. 3, 3–7).

Regulation of formate dehydrogenase Z

It is known that FDH-N synthesis is induced anaerobically in the presence of nitrate. Anaerobic induction requires the *fnr* global regulatory gene and nitrate induction requires *narL* [6]. We wished to examine whether regulation of formate dehydrogenase Z was comparable.

Rocket immunoelectrophoresis was used to monitor the formate dehydrogenase Z content, since the height of the immunoprecipitin arc was proportional to the amount of protein applied as was the case for the FDH-N enzyme (Fig. 1). Our results show that parental strain and *fnr* or *narL* mutants, grown aerobically in the presence or absence of nitrate, all gave enzymati-

cally active precipitin arcs of similar size (Fig. 1). In addition, similar sized immunoprecipitin arcs were found for *narL* and *fnr* mutants regardless of growth conditions. These findings are in good agreement with the enzymatic activities reported in Table II. They demonstrate that unlike FDH-N, formate dehydrogenase Z is constitutively.

We recently identified two genes affecting FDH-N activity in *E. coli* (*fdhD* and *fdhE*). Strains with *fdhD* lesions lack FDH-N activity, but retain essentially normal levels of the protein [25]. Very similar results were obtained with respect to formate dehydrogenase Z which was observed in an equivalent amount to that found in the parental strain regardless of the growth conditions (Fig. 1). Phenazine methosulphate-linked formate dehydrogenase activity was absent in these mutants (Table II).

Formate dehydrogenase Z and nitrate reductase Z can participate in the transfer of electrons from formate to nitrate

FDH-N and nitrate reductase A are specifically induced under anaerobic conditions in the presence of nitrate in *E. coli*. They, with quinones, catalyze electron transfer from formate to nitrate and constitute the formate-nitrate respiratory chain.

We wished to know whether formate dehydrogenase Z can participate in the electron flow from formate to nitrate along with nitrate reductase Z. Experiments were performed using strain LCB2048 (*narA narZ*) which is totally devoid of the polypeptides of nitrate reductases A and Z [23] into which a plasmid carrying the structural genes of nitrate reductase Z could be introduced as required. Cells were grown aerobically in order to prevent synthesis of FDH-N. Since the formate-nitrate reductase respiratory chain does not operate in the presence of oxygen due to organism's preference for oxygen as electron acceptor over nitrate. Prior to assay the cells were shifted to anaerobic conditions (N_2 atmosphere) and chloramphenicol was added to stop further protein synthesis. Rocket immunoelectrophoresis confirmed that formate dehydrogenase Z was present and active (Fig. 1) in untransformed strain LCB2048. This strain lacked formate-to-nitrate reductase activity (Table III). This result was expected since nitrate reductase activity is absent consistent with its *narA narZ* genotype.

When plasmid pLCB14 carrying the *narZYWV* operon encoding nitrate reductase Z was introduced into strain LCB2048, formate-to-nitrate reductase activity was restored to a level similar to that of a wild-type strain grown anaerobically in the presence of nitrate [16].

It appears, therefore, that formate dehydrogenase Z with nitrate reductase Z can form an active formate-nitrate pathway when cells are shifted from aerobic to anaerobic conditions. This pathway is present before formate dehydrogenase N and nitrate reductase A can be induced and may allow the cell to rapidly take advantage of the changed growth conditions. It appears that the level of nitrate reductase Z limits the rate of formate-dependent nitrate reduction under these conditions.

TABLE III

Formate dehydrogenase Z can participate in the formate to nitrate pathway

Strain	Relevant genotype	Formate [a] dehydrogenase	Nitrate [b] reductase	Formate [c] nitrate reductase
LCB333	*narA*	0.18	0.02	10
LCB2048	*narA narZ*	0.18	< 0.01	< 1
LCB2048/ pLCB14	*narA / narZ* $^+$	0.15	0.25	100

[a] Phenazine methosulphate-linked formate dehydrogenase expressed as μmole formate oxidized per min per mg of protein.
[b] Expressed as μmole of nitrate reduced per min per mg of protein.
[c] Expressed as a percentage.

Quinones are required for the formate dehydrogenase Z- and nitrate reductase Z- formate-nitrate pathway

In contrast to other microorganisms, *E. coli* produces ubiquinone and menaquinone whatever the growth conditions [24]. It has been shown previously that aerobic respiratory chains require ubiquinone while the formate-nitrate reductase system, composed of formate dehydrogenase N and nitrate reductase A, uses almost exclusively menaquinone. We constructed mutant strains carrying insertion mutations in the nitrate reductase A and Z structural operons and specific mutations in the ubiquinone and/or menaquinone biosynthetic pathways: AN385$_1$ (*ubi narA narZ*), AN386$_1$ (*men narA narZ*) and AN384$_1$ (*ubi men narA narZ*). Functional nitrate reductase Z was introduced into these strains by transformation with pLCB14 which carries the *narZYWV* structural operon. Formate-dependent nitrate reductase activity following aerobic growth should not therefore be limited by the level of nitrate reductase Z activity (see above). These constructions allowed us to show that formate-nitrate reductase consisting of nitrate reductase Z and formate dehydrogenase Z, can operate with either menaquinone or ubiquinone (Table IV). Indeed, resting cells from aerobically grown mutants AN385$_1$ (*ubi*) and AN386$_1$ (*men*) carrying plasmid pLCB14, produced formate-nitrate reductase activity. This activity was at a relatively high level, representing 80% of that of the parental strain AN387 with plasmid pLCB 14 (Table IV). In contrast, formate-nitrate reductase activity of the double quinone (*men ubi*) mutant AN384$_1$ with pLCB14 was dramatically reduced (Table IV). This

TABLE IV

Quinone requirement for formate dehydrogenase Z- and nitrate reductase Z-formate-nitrate pathway

All strains were grown aerobically. $AN385_1$, $AN386_1$ and $AN384_1$ are mutated in the chromosome in both *narGHJI* and *narZYWV* operons. pLCB 14 carries a functional *narZYWV*.

Strain	Relevant genotype	Formate [a] dehydrogenase	Nitrate [b] reductase	Formate [c] nitrate reductase
AN387/pLCB14		0.15	0.30	100
$AN385_1$/pLCB14	*ubi*	0.14	0.22	80
$AN386_1$/pLCB14	*men*	0.13	0.30	75
$AN384_1$/pLCB14	*ubi men*	0.13	0.22	8

[a] Phenazine methosulphate-linked formate dehydrogenase expressed as μmole formate oxidized per min per mg of protein.
[b] Expressed as μmole of nitrate reduced per min per mg of protein.
[c] Expressed as a percentage.

shows that the formate nitrate pathway in aerobic cells has a non-specific requirement for quinone.

When grown aerobically in the presence of nitrate, *E. coli* couples its respiratory chain preferentially to oxygen, in line with the relative redox potentials of the nitrate and oxygen couples. As mutant $AN385_1$ (*ubi narA narZ*) harbouring pLCB14 lacks ubiquinone, the aerobic respiratory chain cannot work efficiently. We found however that, in such a case, nitrite was produced (50 nmole per mg dry weight) during aerobic growth with nitrate, indicating that *E. coli* may use formate dehydrogenase Z and nitrate reductase Z to mediate electron flow from formate to nitrate, even under aerobic conditions.

Discussion

We have recently demonstrated the existence of a second nitrate reductase Z. Nitrate reductase A and Z isoenzymes share several biochemical and immunological properties while having a distinct regulation [16,17,20].

In this communication, we show that a similar situation exists for the FDH-N enzyme. A second phenazine methosulphate-linked formate dehydrogenase has been identified, formate dehydrogenase Z, whose behaviour is similar to that of nitrate reductase Z with respect to nitrate reductase A.

During fermentative growth conditions (in the absence of nitrate), *E. coli* synthesizes another category of formate dehydrogenase, formate dehydrogenase H, which belongs to the separate formate hydrogenlyase pathway. This enzyme has recently been purified [15]. Although both FDH-N and FDH-H are molybdoenzymes, biochemical studies reported so far do not indicate any further relationships. Like the FDH-N enzyme, formate dehydrogenase Z is a membrane-bound molybdoenzyme which is able to participate in the electron coupling between formate oxidation and nitrate reduction. Its activity with phenazine methosulfate and its subunit composition closely resembles that of FDH-N. These observations allow us to eliminate the possibility that formate dehydrogenase Z is part of the formate hydrogenlyase pathway.

Employing an antiserum directed against purified FDH-N, we demonstrate that formate dehydrogenase Z also is recognized weakly by this antiserum. This immunological study shows that the two enzymes possess a set of similar epitopes. Moreover, the subunit compositions of the two enzymes are similar. Altogether, these results suggest that the two respiratory formate dehydrogenase isoenzymes contain similar peptide domains. We suggest that both *E. coli* phenazine methosulphate-linked formate dehydrogenases have evolved from a common ancestor by duplication. Such a situation has already been substantiated for the two nitrate reductases A and Z which share a level of amino acid identity higher than 70% [18,19].

We have shown that formate dehydrogenase Z is synthesized under all culture conditions tested and is not controlled by either the *fnr* or *narL* gene products. A similar situation has already been described for nitrate reductase Z which is expressed constitutively [16,20]. In contrast, when tested in an *fdhD* mutant, both formate dehydrogenase N and Z proteins were present in normal amounts, although they were devoid of formate dehydrogenase activity. Since the two dehydrogenase isoenzymes appear to be differentially regulated, these findings argue against a role of *fdhD* in formate dehydrogenase synthesis and are in agreement with the suggestion [40] that the *fdhD* gene product acts post-translationally to control formate dehydrogenase assembly or maturation.

The simultaneous existence in *E. coli* of formate dehydrogenase Z, an isoenzyme of FDH-N, and nitrate reductase Z, an isoenzyme of nitrate reductase A, is surprising. We show that formate dehydrogenase Z and nitrate reductase Z can catalyze formate-dependent nitrate reduction following aerobic growth. These constitutively expressed enzymes may allow *E. coli* to rapidly adapt in relation to changes in the environment. Thus, when a sudden shift from aerobiosis to anaerobiosis in the presence of nitrate occurs, the aerobically expressed formate dehydrogenase Z and nitrate reductase Z enzymes could function to mediate electron transfer from formate to nitrate, prior to the synthesis of FDH-N and nitrate reductase A.

Further evidence for this hypothesis comes from the behaviour of mutants defective in ubiquinone production. Our results also demonstrate that quinones are required for the physiological transfer of electrons to nitrate via formate dehydrogenase Z and nitrate reduc-

tase Z. However, in contrast to their anaerobic isoenzymes, formate dehydrogenase Z and nitrate reductase Z can act with either ubiquonone or menaquinone. This peculiarity could be taken to indicate a non-specific ancestral system has been maintained by the bacterium in order to assist the electron transfer to nitrate during anaerobic-aerobic transition. From these data, we conclude that formate dehydrogenase Z and nitrate reductase Z are less specific with respect to quinones than FDH-N and nitrate reductase A, respectively, which only recognize menaquinone.

We show here, using a *ubi* strain, where the electron transfer to oxygen is impaired, that formate dehydrogenase Z and nitrate reductase Z can catalyze during aerobic growth nitrate reduction, as evidenced by nitrite accumulation in the growth medium. In the light of the existence of an aerobic formate dehydrogenase Z in *E. coli*, our previous observations showing that *ubi* mutants are sensitive to chlorate under aerobic conditions in contrast to the wild-type strain [41] can be explained. Both nitrate reductases A and Z have chlorate reductase activity [20]. An aerobic formate-nitrate reductase system would be able to reduce chlorate to chlorite, which is highly toxic to the bacterium. Since aerobic respiration does not function in *ubi* mutants, the formate-nitrate reductase system could provide a pathway for aerobic chlorate reduction.

This secondary formate-nitrate pathway, composed of differentially regulated isoenzymes, is not unique in microorganisms. Similar features have been described in Enterobacteriaceae for hydrogenases and DMSO reductases belonging to distinct pathways, but using identical substrates [42,43].

In a recent communication [21], Sawers and coworkers have identified a third selenopolypeptide in *E. coli* (apart from FDH-N and FDH-H), which is synthesized both aerobically and anaerobically in the presence of nitrate and which correlates strongly with the formate oxidase activity previously described by Pinsent [44]. Its pattern of synthesis and its constitutive expression suggest that the second phenazine methosulphate-linked formate dehydrogenase (FDH Z) described in this report is likely to be the third selenopolypeptide. We are currently working on the isolation of the structural genes encoding formate dehydrogenase Z. Analysis of the gene sequence should be of great interest, particularly if it exhibits homology to that of the FDH-N selenopolypeptide.

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