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Research Letter

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Involvement of formate dehydrogenases in stationary phase oxidative stress tolerance in *Escherichia coli*

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One sentence summary: Formate dehydrogenases were involved in menadione tolerance and survival in stationary phase when *E. coli* cells were grown in the presence of glucose under a microaerobic condition.

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ABSTRACT

Previously, we constructed a series of reduced-genome strains of *Escherichia coli* by combining large-scale chromosome deletions and then tested the sensitivity of these strains to the redox-cycling drug menadione. In this study, we analyzed a deletion that increased menadione sensitivity and discovered that loss of selenocysteine synthase genes was responsible for the strain's reduced tolerance to oxidative stress. Mutants of formate dehydrogenases, which are selenocysteine-containing enzymes, were also sensitive to menadione, indicating that these enzymes are involved in oxidative stress during stationary phase, specifically under microaerobic conditions in the presence of glucose. Among three formate dehydrogenases encoded by the *E. coli* genome, two were responsible for the observed phenotypes: formate dehydrogenase-H and -O. In a mutant of *fdhD*, which encodes a sulfur transferase that is essential for formate dehydrogenase activity, formate dehydrogenase-O could still contribute to oxidative stress tolerance, revealing a novel role for this protein. Consistent with this, overproduction of the electron transfer subunits of this enzyme, FdoH and FdoI, increased menadione tolerance and supported survival in stationary phase. These results suggested that formate dehydrogenase-O serves as an electron transfer element in glucose metabolism to promote oxidative stress tolerance and survival in stationary phase.

Keywords: *Escherichia coli*; stationary phase; oxidative stress; redox-cycling drugs; formate dehydrogenase; glucose toxicity

INTRODUCTION

Chemoheterotrophic bacteria obtain energy by decomposing organic compounds. For example, *Escherichia coli* cells utilize glucose and obtain energy in the form of ATP by oxidizing glucose via glycolysis, a process that also reduces NAD^+ to NADH. Regeneration of NAD^+ from NADH is necessary for continuous energy production. Under anaerobic conditions, NAD^+ regeneration is achieved through fermentation. Along with oxidization of NADH, organic acids such as lactate, acetate and formate are synthesized and excreted into spent fluid. When cells are grown under aerobic conditions, NADH is oxidized to NAD^+ by respiration. When the activity of the respiratory chain is not sufficient to quickly oxidize NADH, oxygen extracts electrons from reduced redox enzymes, resulting in the production of toxic reactive oxygen species (ROS). Especially, microaerobic environment increases the level of ROS production because of the lower level of oxygen availability and the higher NADH/NAD^+ ratio (de Graef *et al.* 1999). The cellular physiology under microaerobic condition is different from that of under aerobic condition or that of under anaerobic condition. For example,

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a high-affinity cytochrome oxidase (CydAB) is highly induced only under microaerobic condition (Tseng, Albrecht and Gunsalus 1996). This enzyme plays an important role for oxidative stress and nitrosative stress by reducing H_2O_2 and nitric oxide, respectively (Giuffrè *et al.* 2014).

Under the anaerobic and microaerobic conditions, a significant amount of formate is produced by pyruvate formate lyase in the step of pyruvate decarboxylation (Alexeeva *et al.* 2000). *Escherichia coli* cells have three formate dehydrogenases (FDHs), FDH-H, FDN-N and FDH-O. All three FDHs contain selenocysteine and the molybdenum (Mo) cofactor, which are required for their formate dehydrogenase activities. FDH-N complex and FDH-O complex are localized in inner membrane, oxidize formate into CO_2 and reduce quinone to quinol. FDH-N is induced under anaerobic condition in the presence of nitrate and functions in anaerobic respiration (Wang and Gunsalus 2003). FDH-O is constitutively expressed at low level and reduces toxic effect of formate, which are produced at low level even in aerobic condition during purine biosynthesis (Sawers 1994; Abaibou *et al.* 1995). FDH-H is induced by formate in the absence of external electron acceptors, and interacts with the hydrogenase 3 or 4 and functions as a formate-hydrogen lyase, which oxidizes formate into CO_2 and reduces proton into hydrogen (Wu and Mandrand-Berthelot 1987). Recently, in *Pseudomonas fluorescens*, formate was proposed to play an important role in the anti-oxidative stress defense by supplying NADPH (Thomas *et al.* 2016). H_2O_2 treatment increases the formate level of cytoplasm and spent fluid, and FDH-NADP is induced and reduces $NADP^+$ into NADPH.

When the activity of the respiratory chain is not sufficient, rapid processing of reducing equivalents is important for cellular survival. In stationary phase, the reducing equivalent used for biosynthesis is decreased, which is important for the survival. Although the cellular physiology at stationary phase has been investigated, there has been identified few genes that are responsible for survival and stress tolerance at stationary phase. It is difficult to isolate mutants that are deficient in survival in stationary phase from among a large number of mutagenized cells because it is necessary to examine the viability of each strain. To address this challenge, in a previous effort to identify the genes involved in oxidative stress tolerance in stationary phase, we examined the menadione sensitivity of a series of reduced-genome strains (Iwadate *et al.* 2011). These 32 strains, which were constructed by combining large chromosomal deletions, lack up to 38.9% of the parental chromosome, corresponding to the deletion of more than 1700 genes. Menadione is a member of the class of redox-cycling drugs, which are reduced by redox enzymes and then oxidize molecular oxygen, resulting in the production of superoxide and hydrogen peroxide (Hassan and Fridovich 1979). The reduced-genome strains were sensitive to menadione, but the sensitivity did not correlate with deletion size (Iwadate *et al.* 2011). Analyses of the difference of menadione sensitivity of the reduced genome strains may lead to the identification of a novel gene involved in oxidative stress tolerance in stationary phase within the large-scale chromosome deletions.

In this study, we re-examined the sensitivity of 14 of the 32 reduced-genome strains to high menadione stress. We analyzed one of the deletions that caused an increase in menadione sensitivity and identified *selAB* as the genes responsible. Furthermore, we showed that FDHs, whose functions depend on *selAB*, contribute to menadione tolerance in stationary phase. We then genetically studied the functions of the responsible FDHs and identified a novel function for these enzymes.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture media and PCR primers

All *Escherichia coli* strains in this study were derivatives of MG1655, and the genotypes are provided in Table S1, Supporting Information. Plasmid pACYC184-*fdoHI* was constructed by ligating a PCR-amplified DNA fragment containing *fdoHI* into the *Eco*RV/*Pvu*V fragment of pACYC184 (Chang and Cohen 1978). The *fdoHI* genes were transcribed by read-through transcription of the *cat* gene on pACYC184. Antibiotic medium 3 (AM3, Becton Dickinson), LB rich medium and M9 minimal medium were used for cell culture. Primers used for the construction of deletion mutations are listed in Table S2, Supporting Information. Deletion mutations were constructed by the Red recombination deletion method (Murphy 1998) and transferred by P1 phage-mediated transduction.

Menadione sensitivity assay with rich medium

Overnight cultures with AM3 were diluted 1/500 into fresh AM3. For the reduced-genome strains, 0.5 ml culture was incubated in a 1.7 ml sample tube with an O-ring (TwistTop Vials, Conical, Sorenson, BioScience, Inc., Cat. No. 12621) for 24 h at 37°C with rotation (one rotation per 40 s). Two microliters of 50 mM menadione solution in ethanol (final concentration, 0.2 mM) or 2 μl ethanol (as a vehicle control) was added to the cultures. For other mutants, 1.5 ml culture was incubated in a 1.7 ml sample tube with an O-ring for 24 h at 37°C with rotation. Fifty microliters of 10 mM menadione solution in ethanol (final concentration, 0.33 mM) or 50 μl ethanol was added to the cultures. The cultures were further incubated for 24 h at 37°C with rotation, serially diluted and plated on 0.1% glucose LB plates. After overnight incubation at 37°C, colony-forming units (CFUs) were determined by colony counting.

Menadione sensitivity assay with minimal medium

Overnight cultures with LB were diluted 1/500 into fresh LB medium. Three milliliters of culture was incubated in a test tube for 24 h at 37°C with reciprocal shaking (130 r.p.m.). Cells were harvested by centrifugation, washed twice with M9 medium without a carbon source and suspended in the same volume of M9 medium containing 0.8% glucose. For the deletion mutants, 6.5 μl of 50 mM menadione solution in ethanol (final concentration, 0.23 mM) or 6.5 μl ethanol (as a control) was added to 1.4 ml cell suspension in a sample tube with an O-ring. The cultures were further incubated for 27 h at 37°C with rotation (one rotation per 35 s). For strains containing multicopy plasmids, 4 μl of 50 mM menadione solution in ethanol (final concentration, 0.14 mM) or 4 μl ethanol as a control was added to 1.4 ml cell suspension in a sample tube with an O-ring. The cultures were incubated for 34 h at 37 °C with rotation (one rotation per 40 s), serially diluted and plated on 0.1% glucose LB plates. After overnight incubation at 37 °C, CFUs were determined by colony counting.

Survival assay in stationary phase

Overnight cultures with LB were diluted 1/500 into fresh LB medium. Three milliliters of the culture was incubated in a test tube for 24 h at 37°C with reciprocal shaking (130 r.p.m.). Cells were harvested by centrifugation, washed twice with M9

medium without a carbon source and resuspended in the same volume of M9 medium containing 0.8% glucose. For microaerobic incubation, 1.4 ml cell suspension was incubated in a 1.7 ml sample tube with an O-ring for 3 days at 37°C with rotation (one rotation per 40 s). For aerobic incubation, 5 ml cell suspension was incubated in a 100 ml flask for 3 days at 37°C with reciprocal shaking (130 r.p.m.). For anaerobic incubation, 30 ml cell suspension was incubated in a glass tube topped with a butyl stopper for more than 3 days at 37°C with reciprocal shaking (130 r.p.m.). The cultures were serially diluted and plated on 0.1% glucose LB plates. After overnight incubation at 37°C, CFUs were determined by colony counting. Chloramphenicol was added at a final concentration of 15 μg/ml as required.

FDH activity assay

Overnight cultures with LB were diluted 1/500 into fresh LB medium. Three milliliters of the culture was incubated in a test tube for 24 h at 37°C with reciprocal shaking (130 r.p.m.). Cells were harvested by centrifugation, washed twice with M9 medium without a carbon source and then resuspended in the same volume of M9 medium containing 0.8% glucose. Fifteen milliliters of the cell suspension was collected in a 15 ml plastic tube and incubated for 24 h at 37°C with rotation (one rotation per 40 s). Cells were harvested by centrifugation, washed twice with 67 μM potassium phosphate buffer and suspended in 1 ml of the same buffer. The cells were lysed by sonication, the cell debris was removed by centrifugation (27 173 × *g*, 5 min) and the resultant cell extract was used for the assay. FDH activity was measured by formate-dependent reduction of dichlorophenolindophenol (DCPI) as described previously (Ruiz-Herrera, Showe and DeMoss 1969), except that 67 μM potassium phosphate buffer was used instead of sodium phosphate buffer. One hundred microliters of the reaction mixture contained 67 μM potassium phosphate buffer, 0.078 mM DCPI, 3 mg/ml PMS and 0.3 mg/ml crude extract. After the absorbance at 600 nm was measured for several minutes, 3.75 μl of 500 mM sodium formate was added and the reduction of the absorbance at 600 nm was measured again. An extinction coefficient of 21 000 M^{-1} cm^{-1} was used for calculations. One unit of activity was defined as the reduction of 1 millimole of formate per min.

RESULTS AND DISCUSSION

Functions of FDHs in menadione tolerance

In our previous work, we examined the menadione sensitivity of reduced-genome strains (strains Δ1–Δ23 and Δ25–Δ33) at 4°C (Iwadate *et al.* 2011). Under these conditions, the drug sensitivity of strains Δ1–Δ14 was not significantly higher than that of the wild type. In this study, to examine these strains more precisely, we tested the menadione sensitivity of strains Δ1–Δ14 at 37°C (Fig. S1a, Supporting Information). The results revealed that strain Δ5 was more menadione-sensitive than strain Δ4. Strain Δ5 was constructed by introducing a large-scale chromosome deletion, deletion unit 2-2 (46 155 bp), into strain Δ4 (Hashimoto *et al.* 2005), suggesting that a gene within this deleted chromosomal region was responsible for the increase in menadione sensitivity. We then constructed partial deletions, introduced them into strain Δ4, and examined the menadione sensitivity of the resultant derivative strains (Fig. S1b, Supporting Information). The menadione sensitivity of strain Δ4 carrying the partial deletion Δ(*ysaB-avtA*) or Δ(*ysaB-ysaD*) was not significantly increased, whereas strains harboring Δ(*yiaT-yibI*), Δ(*yiaT-yibF*) or Δ*selAB* were clearly more sensitive to the drug (Fig. S1b–e, Supporting Information and Fig. 1a). Thus, deletion of *selAB* caused the decrease of menadione tolerance in strain Δ4. Further, when Δ*selAB* was introduced into the wild-type strain MG1655, the menadione sensitivity of the resultant strain was clearly elevated (Fig. 1b). These results demonstrate that *selAB* are involved in menadione tolerance.

The *selA* and *selB* genes encode selenocysteine synthase and a selenocysteyl-tRNA-specific translation elongation factor, respectively. These genes are needed for the incorporation of selenocysteine at the UGA codon during the translation of selenoproteins (Sawers *et al.* 1991). In *Escherichia coli*, there are only three selenocysteine-containing enzymes, FDH-H, FDH-N, and FDH-O. In all of these enzymes, FDH activity depends on *selAB* (Abaibou *et al.* 1995). Therefore, we constructed a triple deletion mutant of the three FDHs (strain ΔFDHs) and examined its menadione sensitivity (Fig. 1b). The menadione sensitivity of the strain ΔFDHs was clearly increased, as were those of the Δ*selAB* and Δ*selAB* ΔFDHs mutants (Fig. 1b). These results indicate that the increase in menadione sensitivity caused to Δ*selAB* resulted from the inactivation of FDHs, and that the FDHs are involved in menadione tolerance in stationary phase under microaerobic conditions.

fdhD-dependent and -independent functions of FDHs in menadione tolerance

Synthesis of active FDHs involves a sulfur transferase, FdhD, which transfers sulfur to Mo-bisPGD (molybdo-bis-pyranopterin guanine dinucleotide), an essential cofactor for FDHs (Arnoux *et al.* 2015). Inactivation of *fdhD* leads to a loss of FDH

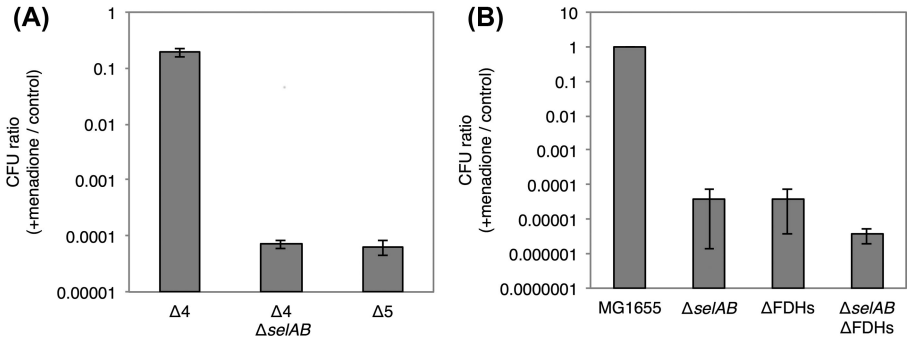

Figure 1. *selAB* and FDHs function in menadione sensitivity in stationary phase under microaerobic conditions in rich medium Antibiotic 3. The cells used in this experiment were the reduced-genome strains Δ4, Δ4 Δ*selAB* and Δ5 **(A)**, and the deletion mutants Δ*selAB* and ΔFDHs **(B)**.

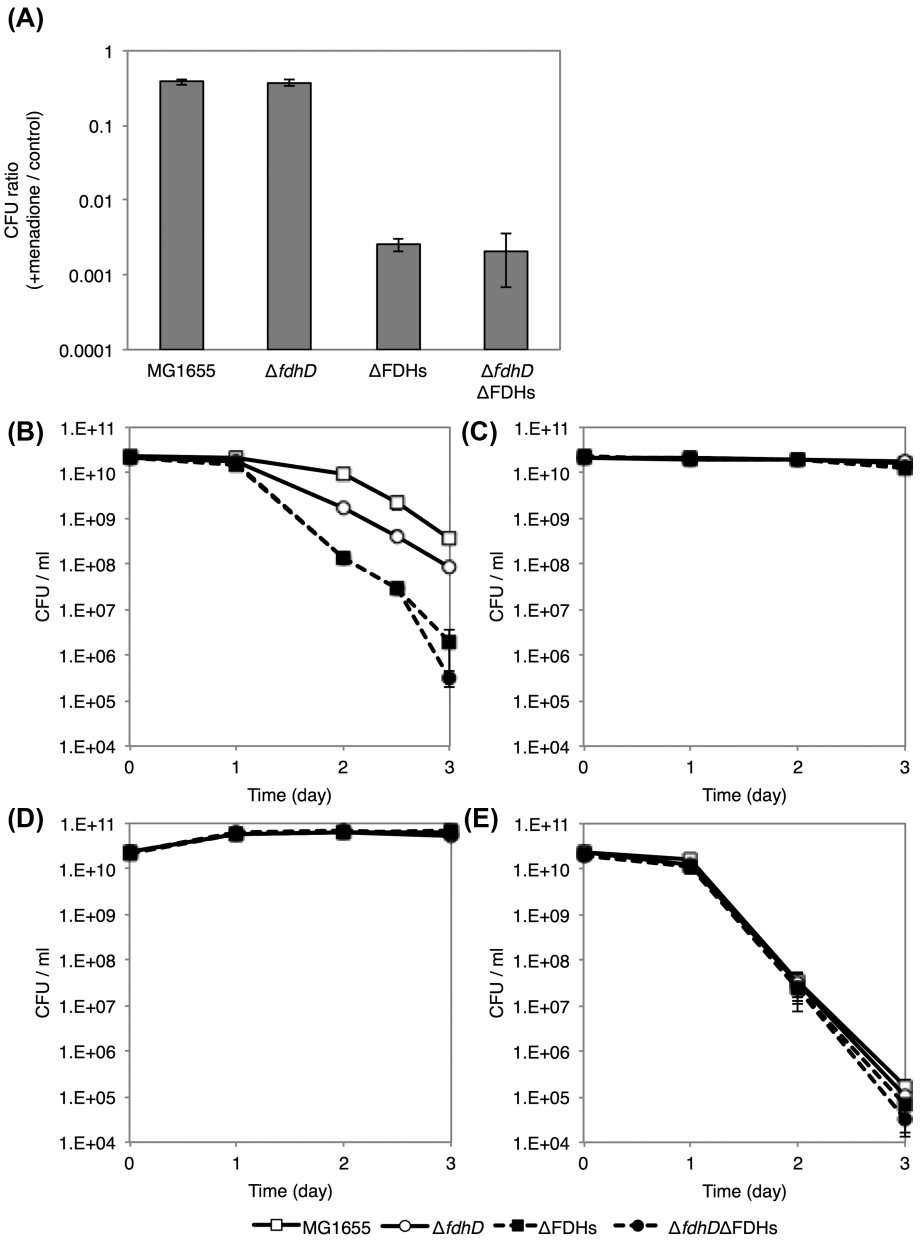

Figure 2. A *fdhD*-independent function of FDHs in the menadione tolerance and survival in stationary phase. Strains were the wild type, Δ*fdhD*, ΔFDHs and Δ*fdhD* ΔFDHs. **(A)** Menadione sensitivity under microaerobic conditions. **(B)** Viability in minimal glucose medium under microaerobic conditions. **(C)** Viability in minimal medium without glucose under microaerobic conditions. **(D)** Viability in minimal glucose medium under aerobic conditions. **(E)** Viability in minimal glucose medium under anaerobic conditions.

activities (Schlindwein *et al.* 1990; Abaibou *et al.* 1995) but does not affect the synthesis of the proteins themselves (Stewart, Lin and Berg 1991). To determine the effects of *fdhD* disruption on menadione sensitivity, we constructed strains Δ*fdhD* and Δ*fdhD* ΔFDHs and tested their tolerance to the drug (Fig. 2a). Unexpectedly, the menadione sensitivity of strain Δ*fdhD* was not significantly increased, whereas that of strain Δ*fdhD* ΔFDHs was increased about 100-fold (Fig. 2a). These results indicated that FDHs function even in the absence of *fdhD*.

Functions of FDHs for survival at stationary phase

When menadione is reduced by redox enzymes, it oxidizes oxygen, yielding ROS (Hassan and Fridovich 1979) and resulting in oxidative stress (Tamarit, Cabiscol and Ros 1998), which decreases survival in stationary phase (Dukan and Nyström 1999). To determine whether menadione-sensitive cells would exhibit reduced viability in stationary phase even in the absence of menadione, we examined the survival of strain ΔFDHs during incubation for 3 days without menadione. The viability of strains ΔFDHs and Δ*fdhD* ΔFDHs was 100-fold lower than that of the wild-type strain, whereas that of strain Δ*fdhD* was only 4-fold lower (Fig. 2b). These results indicated that the FDHs are involved in survival in stationary phase.

To clarify the culture condition under which the FDHs are involved in the survival stationary phase, we examined cells grown under other conditions. When the cells were microaerobically grown in the absence of glucose, the viability of strains

ΔFDHs and Δ*fdhD* ΔFDHs was not much lower than that of the wild-type strain (Fig. 2c). Even in the presence of glucose, when the cells were grown aerobically or anaerobically, the viability of strains ΔFDHs and Δ*fdhD* ΔFDHs was about the same as that of the wild-type strain (Fig. 2d and e). These results showed that the functions of FDHs are necessary for maintaining viability in stationary phase in the presence of glucose under the microaerobic condition.

And to confirm that the phenotype of the FDH mutants was related to oxidative stress, we introduced deletion mutations of the genes for superoxide dismutases into those strains, and then examined the survival of the derivatives in stationary phase. Under our assay conditions, the *sodB* mutation caused the greatest decrease in menadione tolerance (Fig. S2a, Supporting Information), and this mutation had a similar effect on viability in stationary phase (Fig. S2b, Supporting Information). These results suggested that the decrease in viability in stationary phase of strains ΔFDHs and Δ*fdhD* ΔFDHs was due to the elevated level of oxidative stress.

One of the toxicities of menadione depends on ROS, but there were suggested other types of redox stress, direct oxidation of Fe–S cluster (Gu and Imlay 2011) and reversible inactivation of NADH dehydrogenase (Imlay and Fridovich 1992). In this work, we treated cells with menadione in the presence of glucose, then NADH dehydrogenase may not be inactivated. And introduction of the *sodB* deletion caused severe decrease in menadione tolerance, indicating the phenotype is ROS-dependent. Furthermore, the FDH function was necessary for viability in stationary phase even in the absence of menadione. This phenotype was observed under microaerobic condition but not under anaerobic condition. These results suggested that in our experimental conditions the FDH function may be to lessen the ROS production. But FDH may have another function to protect cell from redox stress caused by menadione and further analyses will be necessary.

fdhD-independent function of FDH-O in menadione tolerance and survival in stationary phase

Next, to identify the FDHs involved in survival in stationary phase among the three FDHs encoded by the *E. coli* genome, we constructed three mutant strains, ΔFDH-H ΔFDH-N, ΔFDH-N ΔFDH-O and ΔFDH-O ΔFDH-H, and examined their survival in stationary phase. The viability of strain ΔFDH-O ΔFDH-H was greatly decreased (285.6-fold relative to the wild-type strain), whereas those of strains ΔFDH-H ΔFDH-N (4.0-fold) and ΔFDH-N ΔFDH-O (3.4-fold) were not as dramatically affected (Fig. 3a). These results suggested that FDH-O and -H are involved in survival in stationary phase. In addition, we introduced the *fdhD* deletion mutation into all three mutants and examined their survival in stationary phase. The viability of the strain Δ*fdhD* ΔFDH-H ΔFDH-N was decreased to a lesser extent (4.9-fold relative to strain Δ*fdhD*) than those of strain Δ*fdhD* ΔFDH-N ΔFDH-O (31.3-fold relative to strain Δ*fdhD*) (Fig. 3a). These results suggested that FDH-O, but not FDH-H, had the *fdhD*-independent function.

To determine whether the FDH activity of FDH-O is independent of *fdhD*, we examined the FDH activity of strains ΔFDH-H ΔFDH-N and Δ*fdhD* ΔFDH-H ΔFDH-N. As shown in Table 1, we could detect FDH activity in strain ΔFDH-H ΔFDH-N, but not in strain Δ*fdhD* ΔFDH-H ΔFDH-N. These results were consistent with the previous work (Abaibou *et al.* 1995) confirming that the FDH activity of FDH-O depends on *fdhD*.

The FDH-O complex consists of α, β and γ subunits, which are encoded by *fdoG*, *fdoH* and *fdoI*, respectively. The α subunit,

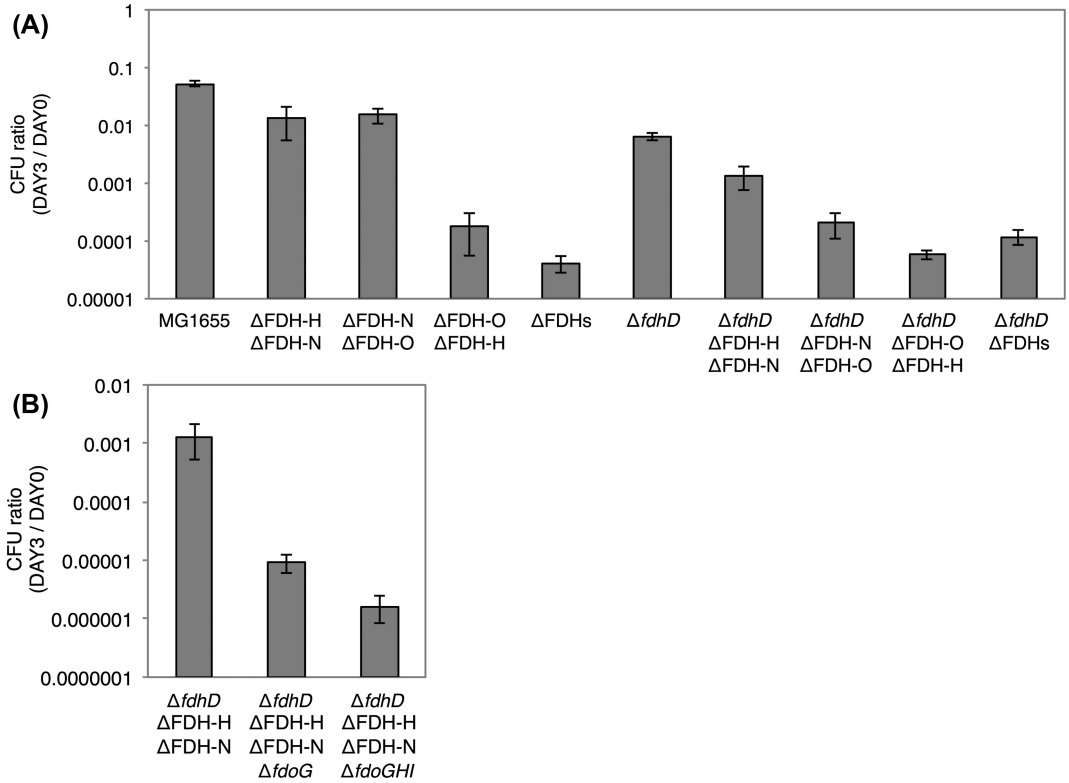

Figure 3. FDH-N and FDH-O function in survival in stationary phase under microaerobic conditions. **(A)** Survival ratio of FDH mutants in stationary phase under microaerobic conditions. **(B)** Survival ratio of mutants containing the *fdoG* or *fdoGHI* deletion mutations in stationary phase under microaerobic conditions.

Table 1. FDH activity of FDH-O in the absence of *fdhD*.

Strain	Units/mg protein
ΔFDH-H ΔFDH-N	1.02 ± 0.03
Δ*fdhD* ΔFDH-H ΔFDH-N	<0.02 ± 0.00
Δ*fdhD* ΔFDHs	<0.02 ± 0.01

One unit of activity was defined as the reduction of 1 millimole of formate per min.

which functions in cooperation with a molybdopterin cofactor, is necessary for FDH activity, and the β and γ are the membrane-bound electron transfer subunits. To confirm that the FDH activity of FDH-O was not involved in survival in stationary phase, we introduced the deletion mutation of *fdoG* into strain Δ*fdhD* ΔFDH-H ΔFDH-N and examined the survival of the resultant derivative in stationary phase. The viability of this mutant was significantly higher than that of strain Δ*fdhD* ΔFDH-H ΔFDH-N Δ*fdoGHI* (Δ*fdhD* ΔFDHs) (Fig. 3b), although the deletion of *fdoG* resulted in 130-fold decrease in viability. These results also suggested that an *fdhD*-independent function of FDH-O is involved in survival in stationary phase.

To further confirm that FDH-O promotes survival in stationary phase in the absence of FDH activity, we examined the effect of overproduction of only the β (FdoH) and γ (FdoI) subunits on viability in stationary phase. For this purpose, we cloned the *fdoH* and *fdoI* genes downstream of the *cat* gene (encoding chloramphenicol resistance) on a multicopy plasmid. When we introduced the plasmid into strain Δ*fdhD* ΔFDHs, both menadione tolerance and the survival in stationary phase were increased (Fig. 4). The results confirmed that FDH-O can function without FDH activity.

Functions of the electron transport system in menadione tolerance and survival in stationary phase

Because the β and γ subunits function in electron transport, the *fdhD*-independent functions of FDH-O may also be related to this process (Fig. 5). To explore this idea, we studied the involvement of the electron transport chain in the downstream of the FDH functions that promote menadione tolerance and survival in stationary phase. First, we examined the NADH dehydrogenase I (Ndh-1) complex, which consists of 13 subunits that contain Fe–S clusters and sequentially pass electrons from NADH to quinone (Efremov and Sazanov 2012). We constructed a deletion mutation of the *nuoABCEFGHIJKLMN* (*nuoA-N*) operon, which encodes the Ndh-1 complex, and examined its effects on phenotype. Both menadione tolerance and survival in stationary phase of strain Δ*nuoA-N* Δ*fdhD* ΔFDHs/pACYC184-*fdoHI* were reduced to the corresponding levels in the control strain Δ*nuoA-N* Δ*fdhD* ΔFDHs/pACYC184 (Fig. S3a and b, Supporting Information). However, the tolerance and viability of strain Δ*nuoA-N* Δ*fdhD* were not reduced to the corresponding levels in control strain Δ*nuoA-N* Δ*fdhD* ΔFDHs (Fig. S3c and d, Supporting Information). These results suggested that Ndh-1 is involved in the increase in menadione tolerance and survival in stationary phase due to overproduction of the FdoH and FdoI subunits, but not the increase due to chromosomal *fdoHI*. Thus, overproduced FdoH and FdoI subunits may interact with the Ndh-1 complex.

Escherichia coli has another NADH dehydrogenase (Ndh-2) encoded by *ndh*. We constructed a deletion mutation of *ndh*, but this mutation did not eliminate the effect of introducing the FdoHI$^+$ plasmid into strain Δ*fdhD* ΔFDHs on menadione tolerance or stationary phase survival (Fig. S3a and b, Supporting Information). Ndh-2 is necessary for respiration with oxygen and nitrate, both of which are high-reduction potential electron acceptors, whereas Ndh-1 is required for respiration with fumarate and DMSO (Tran *et al.* 1997). Ndh-2 is a single membrane-bound polypeptide whose expression is induced under aerobic condition (Green and Guest 1994; Liu and De Wulf 2004). Under the microaerobic condition used in this work, Ndh-2 may be synthesized at very low levels if at all.

Next, we examined the effect of KCN, an inhibitor of cytochrome oxidases. FDH-O forms a supracomplex with the cytochrome bdI or cytochrome bo_3 oxygen reductases. Addition of KCN to the cell suspension decreased survival in stationary phase of the wild-type strain and strain Δ*fdhD* to the levels in control strains ΔFDHs and Δ*fdhD* ΔFDHs (Fig. S4a, Supporting Information). However, the survival of strain Δ*fdhD* ΔFDHs/pACYC184-*fdoHI* was not reduced to the level in the control strain Δ*fdhD* ΔFDHs/pACYC184 (Fig. S4b, Supporting Information). These results suggested that cytochrome oxidases are involved in the survival in stationary phase due to the *fdhD*-independent functions of FDHs, but not in the increase in survival due to overproduction of the FdoH and FdoI subunits, which may not interact with cytochrome oxidases. This is consistent with the observation that survival in stationary phase of strain Δ*fdhD* ΔFDHs/pACYC184-*fdoHI* was not reduced to the level of the control strain Δ*fdhD* ΔFDHs/pACYC184 under the anaerobic condition (Fig. S4c, Supporting Information). The overproduced FdoH and FdoI subunits may interact with quinone oxidases other than cytochrome oxidases. These results suggested

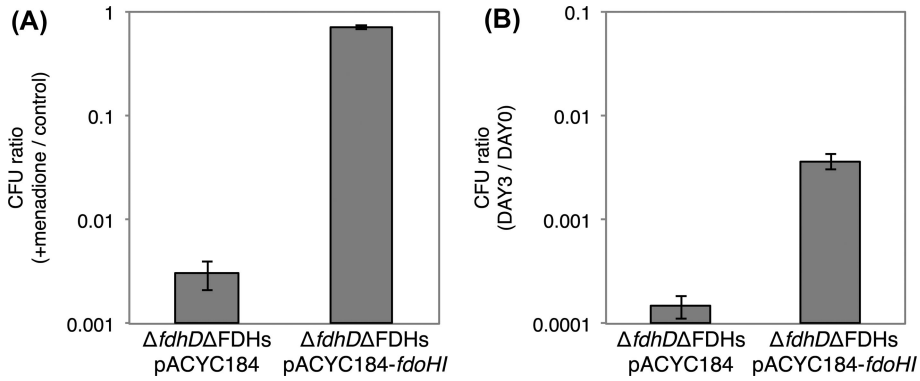

Figure 4. Functions of overproduced FdoH and FdoI subunits of FDH-O in the menadione tolerance and survival at stationary phase. Menadione sensitivity **(A)** and survival at stationary phase **(B)**. Strains ΔFDHs Δ*fdhD* harboring pACYC184 vector or pACYC184-*fdoHI*.

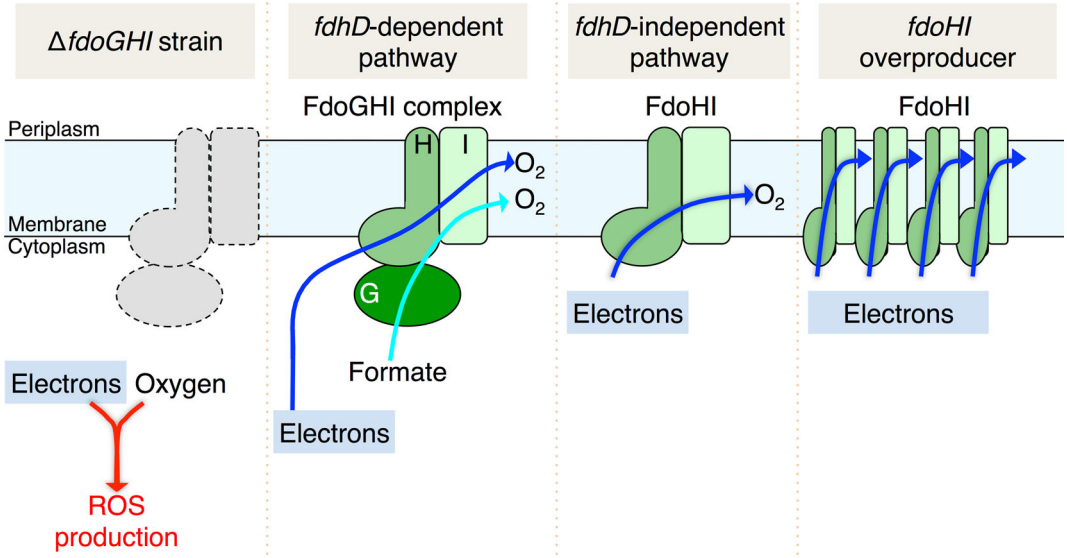

Figure 5. Models of FDH-O functions in oxidative stress tolerance. In strain ΔFDH-O, ROS may be produced by oxygen and reducing equivalents (electrons) synthesized by glucose oxidation under microaerobic conditions. In the wild-type strain, FDH-O may have *fdhD*-dependent and -independent functions in oxidative stress tolerance. Reducing equivalents are transferred to the electron transport chain. In strain Δ*fdoG* or the strain in which FdoH and FdoI are overproduced, FDH-O may have only the *fdhD*-independent function.

that, when present at normal levels, the FdoH and FdoI subunits may accept an electron not from Ndh-1 but from another electron donor and transfer it to cytochrome oxidases, and that overproduced FdoH and FdoI subunits may accept an electron from Ndh-1 and transfer it to quinone oxidases other than cytochrome oxidases. Further biochemical analyses will be necessary to clarify the mechanism of the *fdhD*-independent functions of FDHs in menadione tolerance and survival in stationary phase. These suggest that suitable processing of the reducing equivalents produced by glucose oxidation functions as an indirect response to oxidative stress and is important for survival in stationary phase.

CONCLUSION

FDHs were involved in menadione tolerance and survival in stationary phase when *Escherichia coli* cells were grown in the presence of glucose under a microaerobic condition. Among three FDHs encoded by the *E. coli* genome, FDH-H and FDH-O were responsible for this phenotype, and a subset of FDH-O functions was *fdhD*-independent, suggesting that the enzyme has a novel function independent of dehydrogenase activity. Similarly, artificial overproduction of the electron transfer subunits of FDH-O, FdoH and FdoI rendered cells tolerance to menadione and boosted survival in stationary phase. Redox-balancing activity via electron transfer may be important for repression of endogenous ROS production.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflicts of interest. None declared.

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