

Anaerobic Expression of *Escherichia coli* Succinate Dehydrogenase: Functional Replacement of Fumarate Reductase in the Respiratory Chain during Anaerobic Growth

ELENA MAKLASHINA, DEBORAH A. BERTHOLD,† AND GARY CECCHINI*

Molecular Biology Division (151-S), VA Medical Center, San Francisco, California 94121, and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

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Succinate-ubiquinone oxidoreductase (SQR) from *Escherichia coli* is expressed maximally during aerobic growth, when it catalyzes the oxidation of succinate to fumarate in the tricarboxylic acid cycle and reduces ubiquinone in the membrane. The enzyme is similar in structure and function to fumarate reductase (menaquinol-fumarate oxidoreductase [QFR]), which participates in anaerobic respiration by *E. coli*. Fumarate reductase, which is proficient in succinate oxidation, is able to functionally replace SQR in aerobic respiration when conditions are used to allow the expression of the *frdABCD* operon aerobically. SQR has not previously been shown to be capable of supporting anaerobic growth of *E. coli* because expression of the enzyme complex is largely repressed by anaerobic conditions. In order to obtain expression of SQR anaerobically, plasmids which utilize the P_{FRD} promoter of the *frdABCD* operon fused to the *sdhCDAB* genes to drive expression were constructed. It was found that, under anaerobic growth conditions where fumarate is utilized as the terminal electron acceptor, SQR would function to support anaerobic growth of *E. coli*. The levels of amplification of SQR and QFR were similar under anaerobic growth conditions. The catalytic properties of SQR isolated from anaerobically grown cells were measured and found to be identical to those of enzyme produced aerobically. The anaerobic expression of SQR gave a greater yield of enzyme complex than was found in the membrane from aerobically grown cells under the conditions tested. In addition, it was found that anaerobic expression of SQR could saturate the capacity of the membrane for incorporation of enzyme complex. As has been seen with the amplified QFR complex, *E. coli* accommodates the excess SQR produced by increasing the amount of membrane. The excess membrane was found in tubular structures that could be seen in thin-section electron micrographs.

Succinate dehydrogenase (succinate-ubiquinone oxidoreductase [SQR]) and fumarate reductase (menaquinol-fumarate oxidoreductase [QFR]) are enzyme complexes similar both in composition and in subunit structure, even though in vivo they normally catalyze their enzymatic reactions in opposite directions (2, 11). Each enzyme is a membrane-bound complex that can catalyze a two-electron or two-proton transfer between succinate or fumarate and quinone or quinol. Succinate dehydrogenase plays an important role in cellular metabolism and directly connects the Krebs cycle with the aerobic respiratory chain. Fumarate reductase catalyzes the final step in anaerobic respiration with fumarate as a terminal electron acceptor (21). These enzyme complexes are thus examples of the high evolutionary adaptation of organisms to specialized environments (11).

In *Escherichia coli*, two distinct operons encoding the SQR (*sdhCDAB*) (7, 37) and QFR (*frdABCD*) subunits are found (6, 22). Both enzymes have a large domain extrinsic to the membrane composed of a flavoprotein subunit (SdhA or FrdA) which contains a covalently bound flavin adenine dinucleotide cofactor and the dicarboxylate binding site (2, 11, 27) and an iron-sulfur protein subunit (SdhB or FrdB) containing three

distinct iron-sulfur clusters (17, 20). This domain is bound to two small integral membrane subunits (SdhC and -D or FrdC and -D) which are necessary to form the quinone binding site(s) (4, 36) found in both of these enzyme complexes. One difference between SQR and QFR in *E. coli* is that SQR contains an additional prosthetic group, a heme b_{556} cofactor, which bridges the SdhC and SdhD subunits (33).

It has been 35 years since the first demonstration that in *E. coli* there are two distinct enzyme systems catalyzing fumarate reduction and succinate oxidation (13). The majority of mechanistic studies on succinate dehydrogenase, however, have been done on the mammalian enzyme from bovine mitochondria (2, 12). Kinetic experiments on SQR have shown that the enzyme is much more capable of oxidizing succinate than of reducing fumarate, with the ratio of succinate oxidation to fumarate reduction on the order of 40:1 (9). In contrast, more recent cyclic voltammetry studies of soluble succinate dehydrogenase (SdhAB) adsorbed on a graphite electrode showed that rates of electron transfer between succinate or fumarate and the electrode depended on the applied potential and pH. These results indicated that, at a potential of -75 mV and at pH 7.5, SdhAB functions as proficiently in fumarate reduction as in succinate oxidation (14). As a neutrophilic bacterium, *E. coli* maintains a cytoplasmic pH of 7.6 to 7.8 (23). In the bacterial cell, the composition of the quinone pool is controlled by oxygen. In order to reduce more negative terminal substrates than oxygen under anaerobic conditions, menaquinones (-75 mV) and demethylmenaquinone ($+36$ mV) predominate in the cell membrane (29, 32). Aerobic growth or exposure of cells to oxygen induces ubiquinone ($+112$ mV)

* Corresponding author. Mailing address: Molecular Biology Division (151-S), VA Medical Center-UCSF, 4150 Clement St., San Francisco, CA 94121. Phone: (415) 752-9676. Fax: (415) 750-6959. E-mail: cecchini@itsa.ucsf.edu.

† Present address: Department of Plant Biology, University of Illinois, Urbana, IL 61801.

TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Origin	Genotype	Source or reference
Strains			
MC4100		F ⁻ <i>araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	30
DW35	DW31	<i>zjd::Tn10 Δ(frdABCD)18 sdhC::kan araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	36
HB101		<i>supE44 hsdS20(r_B⁻ m_B⁻) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	26
Plasmids			
pSDH15	pBR322	P _{SDH} <i>sdhC</i> ⁺ <i>D</i> ⁺ <i>A</i> ⁺ <i>B</i> ⁺ Amp ^r	33
pGC1002	pBR322	P _{FRD} <i>frdA</i> ⁺ <i>B</i> ⁺ <i>C</i> ⁺ <i>D</i> ⁺ Amp ^r	4
pH3	pBR322	P _{FRD} <i>frdA</i> ⁺ <i>B</i> ⁺ <i>C</i> ⁺ <i>D</i> ⁺ Amp ^r	3
pH3-177	pACYC177	P _{FRD} <i>frdA</i> ⁺ <i>B</i> ⁺ <i>C</i> ⁺ <i>D</i> ⁺ Amp ^r	This study
pFGS	pBR322	P _{FRD} <i>sdhC</i> ^{+(GTG)} <i>D</i> ⁺ <i>A</i> ⁺ <i>B</i> ⁺ Amp ^r	This study
pFGS-177	pACYC177	P _{FRD} <i>sdhC</i> ^{+(GTG)} <i>D</i> ⁺ <i>A</i> ⁺ <i>B</i> ⁺ Amp ^r Kan ^r	This study
pFAS	pBR322	P _{FRD} <i>sdhC</i> ^{+(ATG)} <i>D</i> ⁺ <i>A</i> ⁺ <i>B</i> ⁺ Amp ^r	This study

production and represses menaquinone biosynthesis (29). Taken together, the above results would indicate that SQR should function as a fumarate reductase under physiological conditions, similar to the demonstrated ability of QFR to function physiologically as a succino-oxidase in cases where SQR function has been disrupted (10).

The control of *sdhCDAB* and *frdABCD* gene expression is provided by two cellular regulatory proteins, ArcA and Fnr, and responds to aerobic-anaerobic conditions and the cellular growth medium (16, 24). Under anaerobic conditions, *sdhCDAB* gene expression is repressed and *frdABCD* expression levels are increased more than 10-fold, whereas aerobic conditions have the opposite effect on SQR and QFR levels (18). Thus, under anaerobic conditions the levels of SQR in *E. coli* are not sufficient to support anaerobic respiration, with succinate dehydrogenase acting in reverse as a fumarate reductase. The present studies were undertaken to determine if in vivo SQR could functionally replace QFR, when conditions are provided to express the *sdhCDAB* genes anaerobically. To accomplish this, vectors in which the P_{FRD} promoter of the *frdABCD* (fumarate reductase) operon was used to express the *sdhCDAB* genes were constructed and the cells were grown anaerobically under conditions requiring fumarate reduction for growth. The results of these studies and the properties of the SQR complex obtained from membranes of anaerobically grown cells are described below.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains and plasmids used in this study are described in Table 1. Strain DW35 (35), a derivative of MC4100, contains a deletion of the *frd* operon (Δ *frdABCD*) and an insertional mutation in *sdh* (*sdhC*::Kan) (36) that eliminates strain background expression of any enzymes capable of succinate oxidase activity. Plasmid pSDH15 encodes the *sdhC*⁺*D*⁺*A*⁺*B*⁺ operon (33), both pH3 and pGC1002 contain the *frdA*⁺*B*⁺*C*⁺*D*⁺ (3, 4) operon, and all are derivatives of pBR322. The construction of plasmids pFGS and pFAS, where the P_{FRD} promoter is used to control expression of the *sdhCDAB* genes, is described below. The latter plasmids are identical, except that pFGS has a GTG initiation codon for *sdhC*, whereas pFAS has an ATG initiation codon in *sdhC*.

Construction of plasmids. Molecular cloning and related procedures were carried out by standard methods (26). Promega kits (Promega Corporation, Madison, Wis.) were used for plasmid isolation. Plasmid pH3-177 was constructed by inserting the 4.44-kb *HindIII-XhoI* fragment of pH3 encompassing the *frd* operon into the *HindIII-XhoI* sites of pACYC177. The construction of plasmid pFGS is diagrammed in Fig. 1. The P_{FRD} promoter was fused to the *sdhCDAB* genes by synthesizing two DNA fragments by PCR and then joining the fragments by the method of PCR overlap extension (15). The P_{FRD} promoter was synthesized with pGC1002 (P_{FRD} *frdA*⁺*B*⁺*C*⁺*D*⁺) as the template with oligonucleotide FRD011 (5'-CACGAATTCAAAGAACGACGG-3') and either FRD012 (5'-CATTCTTATCATGACATTCCTCCAG-3') for plasmid pFAS or oligonucleotide FRD013 (5'-CATTCTTATCAGACATTCCTCCAG-3') for plasmid pFGS used as primer (Fig. 1A). Oligonucleotide FRD011 annealed

3' of the *NdeI* site (5) in the noncoding region of the *frd* operon insert in pGC1002 and encodes an *EcoRI* extension. The first 13 nucleotides of FRD012 and FRD013 were complementary to the promoter sequence immediately 5' of the GTG start codon of *frdA*, and the remaining nucleotides were complementary to the 5' region of *sdhC*. The construction of pFAS was identical to that of pFGS except that oligonucleotide FRD012 was used as a primer in the PCRs in place of FRD013. FRD012 coded for an ATG initiation codon, as is found in wild-type *sdhC*, whereas for pFGS, FRD013 coded for a GTG start codon, as is normally found in *frdA*. A second PCR fragment (II) was synthesized, encompassing the complete *sdhC* gene and the 5' end of the *sdhD* gene, with pSDH15 (*sdhC*⁺*D*⁺*A*⁺*B*⁺) as the template and oligonucleotide ESD001 (5'-TGATAAGAAATGTGAAAAACAAAGAC-3'), which was complementary to the 5' end of *sdhC* (excluding the first nucleotide), and ESD002 (5'-CATTGCGTCTTAA TGCGGAGG-3'), which annealed within *sdhD* (Fig. 1B). The products from the PCRs (Fig. 1A and B, products I and II) were then used as the template for PCR overlap extension with FRD011 and ESD002 as the outside primers (Fig. 1C). The PCR product resulting from the reaction diagrammed in Fig. 1C was then digested with *EcoRI* and *KpnI* (a unique restriction site in the *sdhD* gene) and cloned into the appropriate sites in pSDH15 in order to reconstruct the *sdh* operon under the control of the P_{FRD} promoter (Fig. 1D and E). This region was then sequenced to verify that no inadvertent mutations had been introduced into the coding region. Thus, the resulting plasmid pFGS has a P_{FRD}-*sdhC*⁺*D*⁺*A*⁺*B*⁺ fusion and *sdhC* initiates with a GTG codon. Plasmid pFGS-177 was constructed by inserting the 4.39-kb *AatII-BamHI* fragment of pFGS, encompassing the complete *frd* promoter *sdh* gene region, into the *AatII-BamHI* sites of pACYC177.

Growth conditions. Cells were grown overnight in Luria-Bertani medium with appropriate antibiotics, and then a 1:500 dilution was used as the inoculum for anaerobic minimal medium. Anaerobic growth was carried out at 37°C with constant moderate stirring with a magnet in 1-liter sealed bottles filled to the top with minimal glycerol-fumarate medium (31) supplemented with 0.05% (wt/vol) Casamino Acids and ampicillin (100 µg per ml). Fumarate and glycerol were used at 25 mM concentrations. Growth was monitored by determining cell optical density at 600 nm with a Uvikon 10 spectrophotometer (Kontron Instruments, Zurich, Switzerland).

Thin-section electron microscopy. Cells were collected by centrifugation after 60 h of anaerobic growth and fixed with 2.7% glutaraldehyde–0.8% paraformaldehyde–0.2 M sodium cacodylate (pH 7.2). They were then washed with phosphate-buffered sucrose, postfixed in 2% OsO₄, reduced with 1.5% potassium ferricyanide, and block stained in 2% uranyl acetate. The samples were dehydrated in ethanol and embedded in Spurr's resin (Pelco, Inc., Redding, Calif.). Sections 70 nm thick were poststained with uranyl acetate and lead citrate and mounted on grids for electron microscopy.

Preparation of membrane fraction and enzyme purification. Cells from 1 liter of culture were collected by centrifugation at 4,500 × g for 10 min, then suspended with 200 ml of 100 mM potassium phosphate–5 mM EDTA (pH 7.6), centrifuged again as described above, and then frozen at –70°C. Cells were then suspended in 40 ml of the same buffer containing the "Complete" protease inhibitor tablets (Boehringer Mannheim, Indianapolis, Ind.) and disrupted by sonication. Unbroken cells were removed by centrifugation (15 min at 10,000 × g), and the supernatant was centrifuged for 90 min at 100,000 × g to collect the membranes. The membrane fraction was suspended in 30 ml of the same buffer, and both centrifugation steps were repeated. Membranes were suspended in 50 mM potassium phosphate–0.2 mM EDTA (pH 7.8) to approximately 15 mg of protein/ml and frozen at –70°C. SQR was purified according to a published procedure (19). The purification of QFR was essentially the same as that for SQR, except that a linear gradient of 0.1 to 0.2 M NaCl was used for the DEAE fast-flow chromatography step.

Measurement of enzymatic activity and analytical procedures. Membranes were thawed and diluted with 50 mM potassium phosphate–0.2 mM EDTA (pH

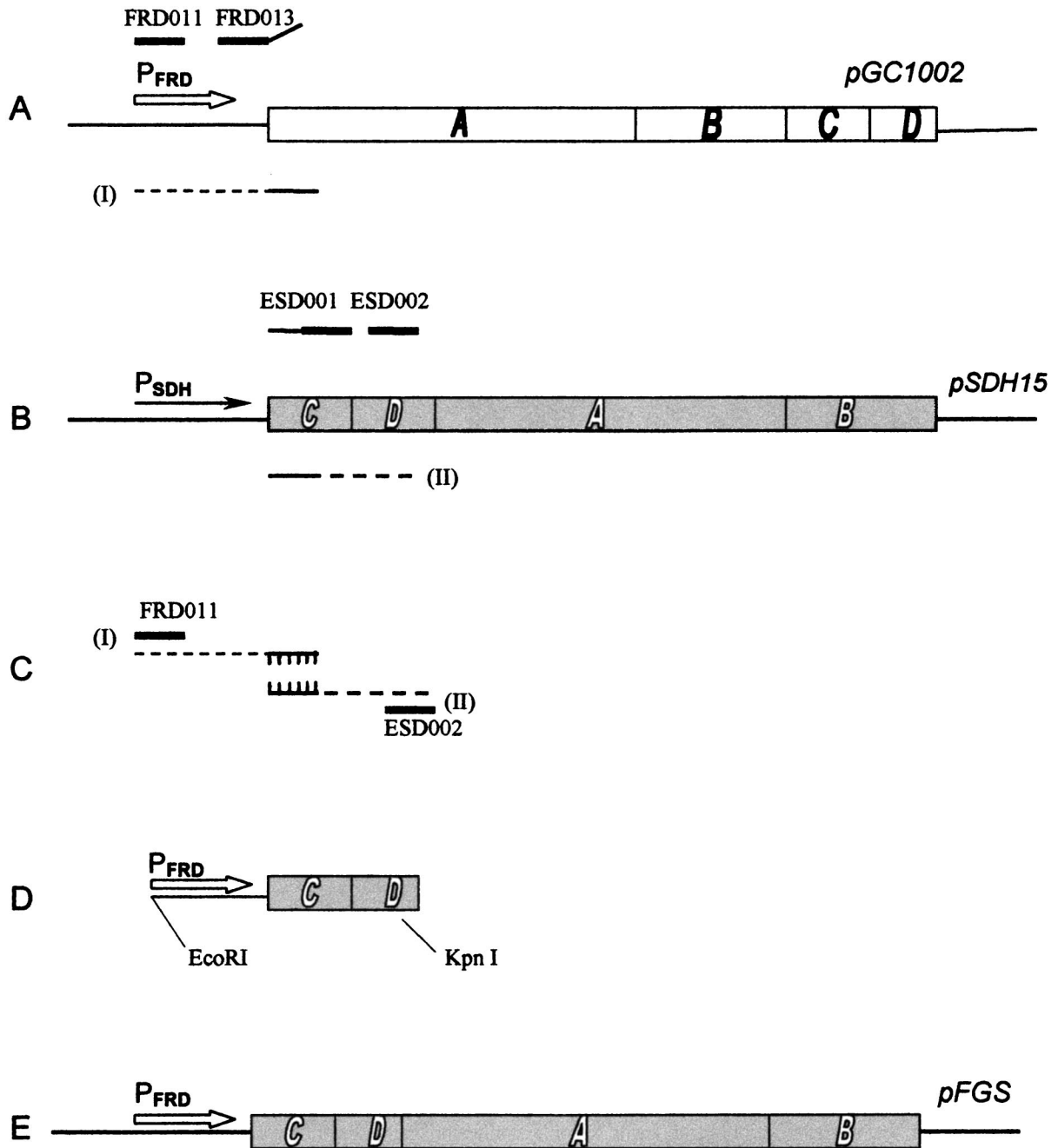


FIG. 1. Schematic diagram showing relevant features of construction of plasmid pFGS. The methodology is described in Materials and Methods. The P_{FRD} promoter is indicated by the open arrow, and the P_{SDH} promoter is indicated by the solid arrow. Oligonucleotide primers used in PCRs are indicated by thick solid lines. The resulting PCR products I and II are indicated by the dashed lines and, as shown in panel C, were joined by the method of PCR overlap extension (15). The *frd* genes in pGC1002 are indicated by solid lettering (A), whereas the *sdh* genes are indicated by open lettering (B). The *Eco*RI and *Kpn*I restriction sites used to create the constructs are indicated.

7.8) to a concentration of 1 mg/ml. In order to measure the full activity of SQR and QFR, it was necessary to remove bound oxaloacetate from the active site of the enzymes. Therefore, 10 mM malonate was added and samples were incubated for 15 min at 37°C and stored at 4°C until used. All enzyme assays were carried out at 30°C in 2-ml cuvettes with 50 mM potassium phosphate–0.2 mM EDTA (pH 7.8)–3 mM KCN. Measurement of succinate oxidation by phenazine ethosulfate (PES) and ubiquinone-2 (Q_2) in the presence of dichlorophenolindophenol ($\epsilon^{600} = 21.8 \text{ mM}^{-1} \text{ cm}^{-1}$; pH 7.8) was performed as previously described (1). The substrates were used at the following concentrations: 10 mM succinate (pH 7.0), 50 μM dichlorophenolindophenol, and 1.5 mM PES or 20 μM Q_2 . The succinate-ferricyanide reductase activity of QFR was determined

with 0.45 mM potassium ferricyanide ($\epsilon^{420} = 1 \text{ mM}^{-1} \text{ cm}^{-1}$). The quinol-fumarate reductase reaction was assayed in a coupled system with rat liver NADH-quinone reductase (DT Diaphorase), with Q_2 and menaquinone-1 (MQ_1) as described elsewhere (9). The succinate-quinone reductase and quinol-fumarate reductase reactions of purified complexes were determined in the presence of 0.005% (wt/vol) of the nonionic detergent Thesit (Boehringer Mannheim).

Absorption spectra were recorded at room temperature by using a diode array rapid-scanning spectrophotometer (8451A; Hewlett-Packard, Palo Alto, Calif.) in a 1-ml anaerobic cuvette. The spectrum of cytochrome b_{556} attributed to SQR was determined essentially as previously described (19) in 50 mM potassium

TABLE 2. Succinate-quinone reductase and quinol-fumarate reductase activity catalyzed by isolated *E. coli* SQR^a and QFR^b (pH 7.8, 30°C)

Quinone	Turnover no. (s ⁻¹) catalyzed by:			
	SQR		QFR	
	Succinate-quinone reductase	Quinol-fumarate reductase	Succinate-quinone reductase	Quinol-fumarate reductase
Q ₂	80		27	
MQ ₁	<0.2		11	
Q ₂ H ₂		2		<0.2
MQ ₁ H ₂		4.8		170

^a SQR was isolated from aerobically grown DW35 transformed with pSDH15.

^b QFR was isolated from anaerobically grown DW35 transformed with pH3.

phosphate–0.2 mM EDTA (pH 7.8) at a protein concentration of 0.25 mg/ml. To subtract the background of heme associated with *bd* oxidase, the distribution of this heme was measured in the presence of 0.5 mM *N,N,N,N*-tetramethyl-4-*p*-phenylenediamine (TMPD) and 0.5 M ascorbate (pH 7.0). Cytochrome *b*₅₅₆ ($\epsilon^{558-575} = 22.8 \text{ mM}^{-1} \text{ cm}^{-1}$) associated with SQR was reduced with 10 mM succinate and sodium dithionite. Protein content was determined by the biuret method with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The rates of quinone reduction and quinol oxidation catalyzed by SQR and the QFR isolated and purified from aerobically and anaerobically grown cells, respectively, are shown in Table 2. It can be seen that the potential of the quinones affected the maximal rates of the reactions. The low-potential donor MQ₁H₂ was a better substrate for fumarate reduction. With the higher-potential acceptor Q₂, the enzymes demonstrated higher rates of succinate oxidation. The data show that SQR catalyzes succinate oxidation by ubiquinone 40 times faster than it does fumarate reduction by ubiquinol (Q₂H₂). SQR catalyzed the reduction of fumarate 2.4 times faster with MQ₁ than Q₂. Moreover, when the enzyme operates with MQ₁ it behaves as a fumarate reductase; the rate of fumarate reduction is more than 20 times higher than that of succinate oxidation. The activity of isolated QFR with quinone-quinols shows the opposite effects from that seen with SQR with respect to quinone potential. Thus, the data support the observation that QFR can functionally replace SQR when expressed aerobically (10). The data in Table 2 thus provide evidence that in vivo SQR should be able to support anaerobic respiration, assuming that conditions are found where the enzyme complex is able to be synthesized.

The first gene in the *frd* operon (*frdA*) belongs to the small group of genes in *E. coli* which use a GUG initiation codon instead of the more common AUG (5). In order to investigate the ability of SQR to support anaerobic growth of *E. coli*, a P_{FRD}-*sdhCDAB* fusion was constructed, with the P_{FRD} promoter driving expression of the *sdhCDAB* genes. Constructs were made with either ATG (pFAS) or GTG (pFGS) as the start codon in *sdhC* and then introduced into vector pBR322 or pACYC177. Initial growth studies using anaerobic minimal glycerol-fumarate medium demonstrated that DW35 (Δ *frd* *sdhC::kan*) transformed with plasmid pFGS would grow with a doubling time of about 3.0 h after a long lag phase (data not shown). In order to reduce this lag phase, the medium was supplemented with varying amounts of tryptone and yeast extract. It was found that 0.025% (wt/vol) tryptone and yeast extract were sufficient to stimulate growth without allowing strain DW35 to grow anaerobically on glycerol-fumarate me-

dium in the absence of QFR- or SQR-encoding plasmids. This medium was used for all further studies.

The results of anaerobic growth are depicted in Fig. 2. To maintain the plasmid in the cell, antibiotics were always present in the growth medium and the growth rates and final culture density were highly reproducible over numerous experiments. DW35, like the wild-type strain MC4100, grew with a doubling time of approximately 1.6 to 1.8 h when transformed with the wild-type QFR-encoding plasmid pH3 or pH3-177. Additionally, the SQR-encoding plasmid pFGS or pFGS-177 allowed the *E. coli* strain to grow with a 3.0- to 3.3-h doubling time, and the final cell density was approximately the same as that with the *frdABCD* plasmids. As a control, DW35 transformed with pSDH15 (*sdhCDAB* driven from the P_{SDH} promoter in pBR322) was grown anaerobically. It can be seen that some growth is possible, although the doubling time is significantly slower than that with the pH3 or pFGS plasmid. It has been shown that the P_{SDH}-*lacZ* fusions can be expressed anaerobically at about 3 to 5% of their aerobic expression level (28), and thus, this low-level expression from a multicopy plasmid with the P_{SDH} promoter is able to support growth with a doubling time of greater than 8 h. The DW35/pSDH15 culture reproducibly stops growing at an optical density at 600 nm of about 0.35 after 48 h of growth, in contrast to results seen with other plasmids. As seen in Fig. 2, cells transformed with the SQR-encoding pFAS plasmid (*sdhC* initiates with an ATG codon) also supported growth but at a significantly slower doubling time of approximately 7.6 h, and the final cell density reached was only about half of that with the pFGS or pFGS-177 plasmid. A similar pattern of inhibition of growth by pFAS was also observed for anaerobically grown cultures on rich medium such as Terrific Broth (26) (data not shown). Aerobic cultures grown on either rich or minimal medium, however, do not show any inhibition by pFAS. The specific reason for inhibition of anaerobic growth by pFAS remains unknown; however, it may reflect the high level of amplification of the membrane-bound SQR from this plasmid (see below). It is possible that the overproduction of membrane-bound proteins from multicopy plasmids may be deleterious to host strains or that growth in the presence of pFAS may deplete the cultures of necessary nutrients for efficient growth. It should be noted, however, that this was not observed for overproduction of QFR in the experiments reported here or by others (2, 8, 34) or for SQR from pFGS (Fig. 2).

The levels of SQR expression were monitored by measuring succinate dehydrogenase activities and heme *b*₅₅₆ content in the membrane fraction of cells grown anaerobically (Table 3). The data show that the enzyme has the same turnover number regardless of the plasmid source used for its expression. Based on the amount of SQR-specific heme *b*₅₅₆ produced from the P_{FRD} promoter with pFGS, the data suggest that succinate dehydrogenase is amplified to the same levels as wild-type QFR from similar vectors. In addition, the SQR complex isolated and purified from both aerobically and anaerobically grown *E. coli* showed no differences in its kinetic properties, rate of heme reduction, composition, and/or stability compared with aerobically expressed wild-type SQR (data not shown). Based on the level of heme *b*₅₅₆ in the membrane, it can be seen in Table 3 that the anaerobic expression of the SQR complex is some seven- to ninefold higher with plasmid pFGS or pFAS, respectively, than when expressed from its own promoter (pSDH15). It should also be noted that, based upon heme content, the levels of SQR produced from anaerobically grown *E. coli* containing pFAS or pFGS are approximately twofold higher than that found in aerobic cells where SQR (pSDH15) is expressed from its native promoter (Table 3).

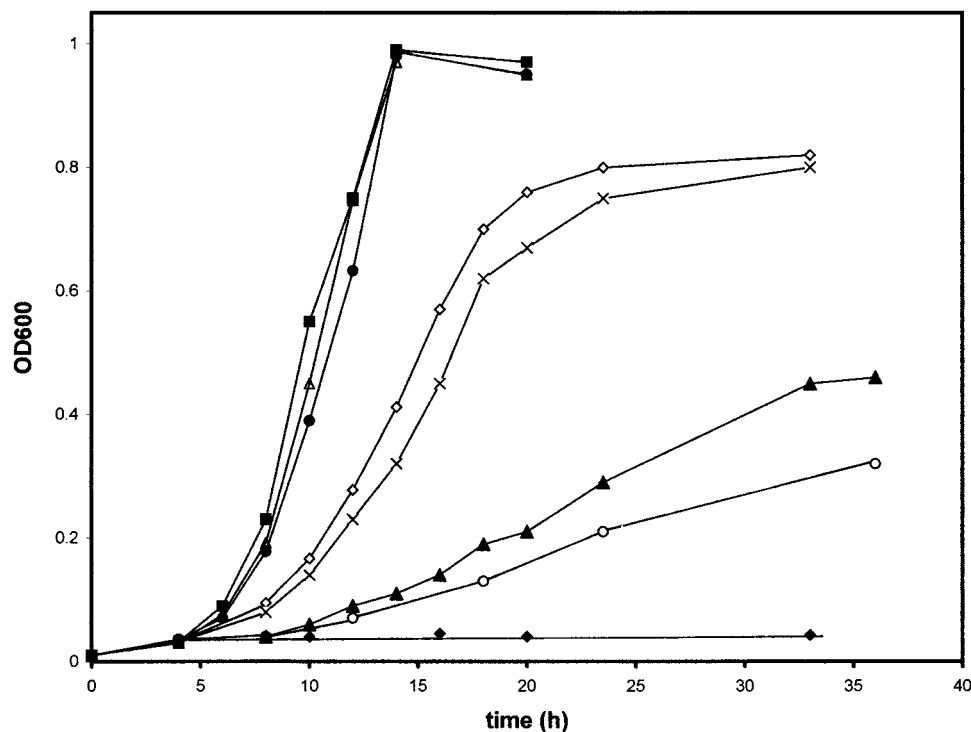


FIG. 2. Anaerobic cell growth. DW35 (Δ frd *sdhC::kan*) cells carrying pBR322 (Amp^r) (\blacklozenge), pH3 (P_{FRD} *frdABCD* in pBR322) (\bullet), pH3-177 (P_{FRD} *frdABCD* in pACYC177) (Δ), pFGS (P_{FRD} *sdhC^{GTG} DAB* in pBR322) (\diamond), pFGS-177 (P_{FRD} *sdhC^{GTG} DAB* in pACYC177) (\times), pFAS (P_{FRD} *sdhC^{ATG} DAB* in pBR322) (\blacktriangle), and pSDH15 (P_{SDH} *sdhCDAB* in pBR322) (\circ) and strain MC4100 (wild type) with pBR322 (\blacksquare) were grown on glycerol-fumarate medium supplemented with 0.025% tryptone and 0.025% yeast extract as described in Materials and Methods. OD600, optical density at 600 nm.

This result is supported by the observation that a three- to fivefold-higher yield of purified SQR enzyme complex is obtained from an equivalent cell mass of anaerobically grown *E. coli* containing either pFAS or pFGS than from aerobically grown cells containing pSDH15 (data not shown).

In the present study, *E. coli* succinate-quinone oxidoreduc-

tase, normally a component of the aerobic respiratory chain, was expressed during anaerobic cell growth on glycerol-fumarate medium by using the P_{FRD} promoter to achieve expression. Under these conditions where quinol-fumarate reductase activity is essential for respiration, SQR functionally replaced QFR in the simplified anaerobic respiratory chain consisting of

TABLE 3. Specific activity and heme contents of membranes obtained from the anaerobically grown cells^a

Strain or plasmid	Succinate-acceptor reductase activity (2 electron equivalents/min/mg of protein) (10^{-6})			Heme b_{556} ^b (nmol/mg)	Turnover number ^c (S^{-1})	Amplification level ^d	Doubling time (h)
	PES	$\text{K}_3\text{Fe}(\text{CN})_6$	O_2				
<i>frdABCD</i> plasmid							
MC4100 (wild type)	0.6	0.6	0.3		25	1	1.6
pH3-177	6.1	6.6	3.3		27	11	1.6
pH3	7.6	9.4	4.6		28	15	1.8
<i>sdhCDAB</i> plasmid							
pFGS-177	14	— ^f	12.8	2	102	11	3.3
pFGS	18.8	—	17.5	2.7	105	15	3.0
pFAS	24	—	22	3.6	102	19	7.6
pSDH15	2.7	—	2.3	0.4	96	2	>8
Aerobically grown cells ^e (pSDH15)	10.5	—	9.4	1.5	100	ND ^g	ND

^a All cells were DW35 except the MC4100 wild type.

^b Determined as level reduced with dithionite minus level reduced with ascorbate-TMPD.

^c Based on succinate- O_2 reductase reaction.

^d Compared to wild-type expression of QFR in MC4100.

^e Cultures were grown aerobically on the same medium as anaerobically grown cultures except that 25 mM succinate replaced fumarate.

^f SQR does not show succinate-ferricyanide reductase activity.

^g ND, not determined.

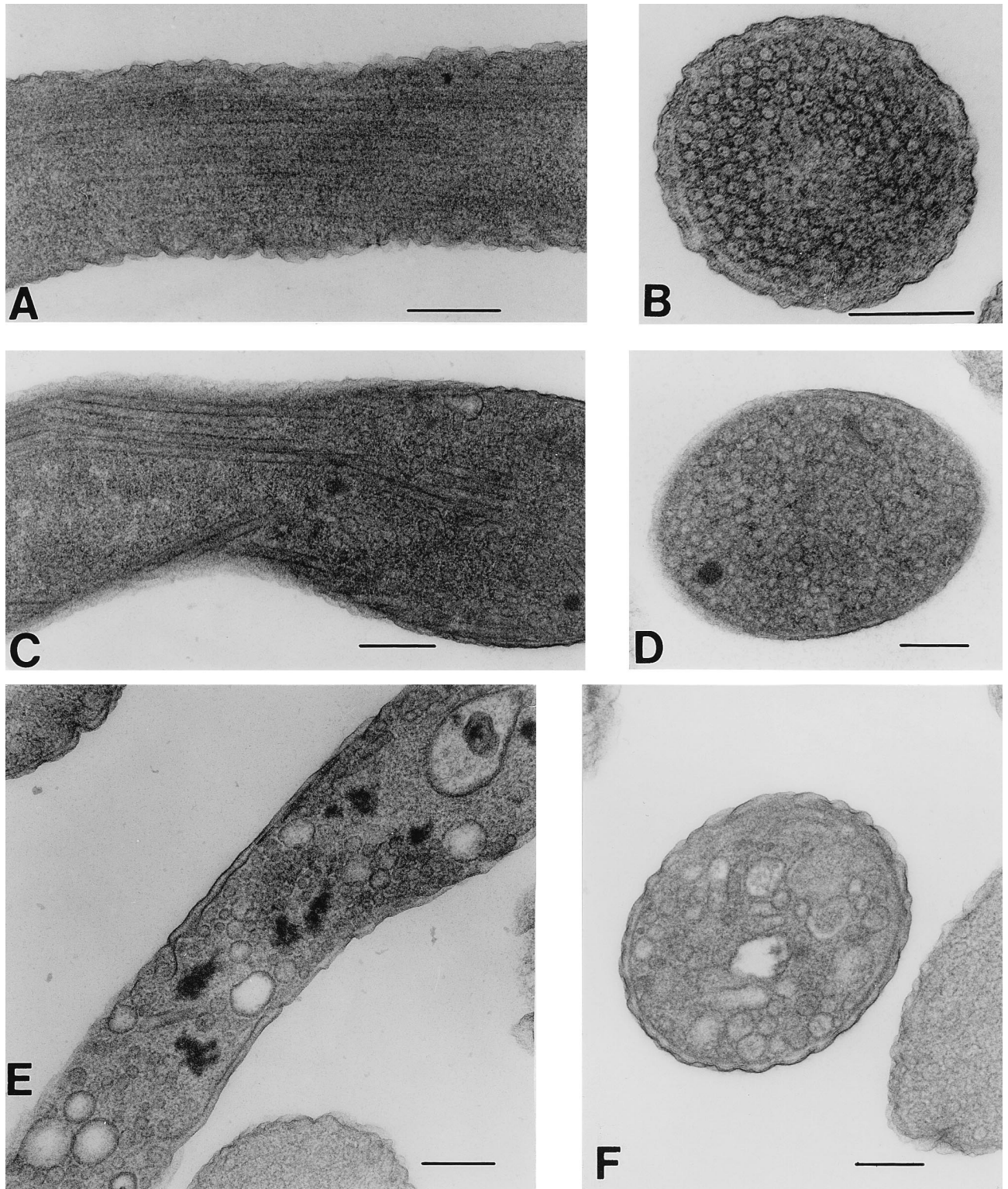


FIG. 3. Thin-section electron microscopy of *E. coli*. Cultures were grown to stationary phase as described in Materials and Methods. (A and B) Longitudinal and lateral sections, respectively, of HB101/pH3 expressing fumarate reductase. (C to F) DW35/pFAS cells expressing SQR complex: (C and D) tubule formation; (E and F) cytoplasmic vesicle formation from amplified expression of SQR. Bars, 0.2 μm .

the anaerobic glycerol-3-phosphate dehydrogenase and menaquinones. Previous work from the Guest laboratory (10) has shown that fumarate reductase can offset the metabolic consequences of a deficiency of succinate dehydrogenase and thus

replace the physiological function of succinate dehydrogenase. This was achieved by allowing amplification of fumarate reductase production aerobically through titration of a specific repressor (10). A similar observation was made when *E. coli*

DW35 was grown with plasmid pH3 or pGC1002 on aerobic succinate minimal medium (3, 27, 36). The results described in this work directly demonstrate that the converse can occur, i.e., succinate dehydrogenase can physiologically replace fumarate reductase when conditions allow it to be expressed anaerobically. These results are also consistent with *in vitro* studies, using soluble beef heart succinate dehydrogenase, which suggest that the soluble enzyme (SdhAB domain) is physiologically capable of catalyzing fumarate reduction at pH values below 7.64 (14) and support the contention that complex II will also function as a fumarate reductase.

In spite of a significant difference in turnover numbers of menaquinol-fumarate reductase reaction of SQR and QFR (Table 2), the cells encoding succinate dehydrogenase from pFGS and pFGS-177 grew only about two times slower (Fig. 2 and Table 3) than cells with *frdABCD*. In the case of QFR, up to 15-fold amplification of enzyme levels did not affect the culture's doubling time during anaerobic growth. These results suggest that the levels of fumarate reductase are not in themselves rate limiting for cell growth under the conditions tested. As shown in Table 3, the 10- to 15-fold overproduction of SQR obtained from plasmid pFGS and pFGS-177 is similar to the overproduction obtained from the QFR plasmids pH3-177 and pH3, which were constructed from the same vectors. This difference in SQR amplification in the membrane did not affect growth, as seen from the similar doubling times. It is significant to note, however, that although the level of enzyme produced in the membrane from plasmid pFAS is about 30% higher than that from pFGS, growth was impaired from this plasmid (Fig. 2). It has been found that the translation efficiency of *E. coli* proteins depends on the initiation codon, and the level of gene product is increased by changing the first codon from GUG to AUG (25). Furthermore, it has been shown that a promoter fusion, P_{FRD} -*lacZ*⁺, starting with the ATG codon, gave a five-fold-higher expression level than that with protein fusion *frdA'*-*lacZ*⁺, which starts from a GTG codon (18). The data presented here are consistent with the higher level of expression when translation of the *sdh* genes begins with the AUG codon.

It has previously been shown that, in stationary-phase *E. coli* cells harboring a fumarate reductase-encoding plasmid similar to pH3, QFR production was amplified 20-fold (34). Because of the limited capacity of the inner membrane, the cells produced novel tubular structures rather than accumulating the extra protein in soluble form, or as inclusion bodies. The lipid-protein tubular structure was composed of QFR and enriched for cardiolipin compared to normal membranes (8, 34). Since SQR and QFR are similar in composition and function and because they are being expressed under similar growth conditions, it seemed likely that similar structures would be formed in cells containing the P_{FRD} -*sdhCDAB* expression system. As shown in Fig. 3, structures resembling the QFR tubules are formed in DW35 transformed with pFAS when cells are grown anaerobically on glycerol-fumarate medium. While QFR overproduction induced the formation of tubular structures in the cytoplasm (Fig. 3A and B) (8, 34), cells harboring pFAS showed both tubular structures and vesicle-like structures in the cytoplasm (Fig. 3C to F). These differences are reproducible; however, the reason for the difference in type of structure formed is unknown. These structures are seen in either DW35 or a *frd*⁺ *sdh*⁺ strain of *E. coli* such as HB101. The overproduction of SQR causes tubule formation with either plasmid pFGS (not shown) or pFAS; however, it is more prevalent with the latter. It should also be noted that pFAS inhibits the growth rate of HB101 (similar to DW35 [Fig. 1]) even though this strain contains a wild-type copy of *frdABCD*. The significant decrease in cell doubling time seen in cultures containing

pFAS may thus reflect the depletion of the resources necessary for efficient growth by enzyme overproduction.

The results described in this work are the first direct demonstration that succinate dehydrogenase can physiologically replace fumarate reductase when conditions allow it to be expressed anaerobically. An additional finding is that the isolated SQR complex obtained from anaerobically grown *E. coli* is produced in a fully active form and at a greater yield in the membrane (based on heme content) than aerobically produced enzyme. This is an aid in obtaining material for biochemical investigation of *E. coli* succinate dehydrogenase. By using the P_{FRD} -*sdhCDAB* fusion with single-copy vectors, it should now also be possible to isolate SQR mutants that are altered in their ability to grow anaerobically. Questions remain as to the cause of the differences in catalytic activity seen with QFR and SQR; therefore, isolation of mutant forms of both enzyme complexes under physiological growth conditions will aid in understanding these differences.

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