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*frdD* 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 enzyme 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.
plasmid pFGS is diagrammed in Fig. 1. The P\text{FRD} promoter was fused to the \text{frd} genes by synthesizing two DNA fragments by PCR and then joining the fragments by the method of PCR overlap extension (15). The \text{FRD} promoter was synthesized with pGCl002 (P\text{FRD} frdA’ B’ C’ D’ Amp’). The PCR product resulting from the reaction diagrammed in Fig. 1C was then annealed with SDH011 and ESD002 as the outside primers (Fig. 1C). The PCR product resulting from the reaction and shown in Fig. 1C was then digested with EcoRI and KpnI (a unique restriction site in the sdhC gene) and cloned into the appropriate sites in pSDH15 in order to reconstruct the sdh operon under the control of the P\text{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\text{FRD} sdhD C’ D’ A’ B’ fusion and sdhC initiates with a GTG codon. Plasmid pFGS-177 was constructed by inserting the 4.39-kb \text{AatII}-\text{ BamHI} fragment of pFGS, encompassing the complete \text{frd} promoter sdh region, into the \text{AatII}-\text{ 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 Casamino Acids and ampicillin (100 μg/ml). Cells were collected by centrifugation after 48 h of growth. Thin-section electron microscopy. Cells were fixed in 4% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2). They were then washed with phosphate-buffered sucrose, postfixed in 2% OsO₄ reduced with 1.5% potassium ferrocyanide, 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. The membrane fraction was suspended in 30 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 7.6) to approximately 15 mg of protein/ml and frozen at −70°C. The membranes were 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 7.6).
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 (Q2) in the presence of dichlorophenolindophenol (ε600 = 21.8 mM⁻¹ 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 Q2. The succinate-ferricyanide reductase activity of QFR was determined with 0.45 mM potassium ferricyanide (ε420 = 1 mM⁻¹ cm⁻¹). The quinol-fumarate reductase reaction was assayed in a coupled system with rat liver NADH-quinone reductase (DT Diaphorase), with Q2 and menaquinone-1 (MQ1) 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 b₅₅₆ attributed to SQR was determined essentially as previously described (19) in 50 mM potassium phosphate–0.2 mM EDTA–3 mM KCN–50 mM potassium ferricyanide–1 mM PES (pH 7.8). The quinol-fumarate reductase reaction was assayed in a coupled system with rat liver NADH-quinone reductase (DT Diaphorase), with Q2 and menaquinone-1 (MQ1) 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).

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FIG. 1. Schematic diagram showing relevant features of construction of plasmid pFGS. The methodology is described in Materials and Methods. The P<sub>FRD</sub> promoter is indicated by the open arrow, and the P<sub>SDH</sub> 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 <i>fdl</i> genes in pGC1002 are indicated by solid lettering (A), whereas the <i>sdh</i> genes are indicated by open lettering (B). The EcoRI and KpnI restriction sites used to create the constructs are indicated.
TABLE 2. Succinate-quinone reductase and quinol-fumarate reductase activity catalyzed by isolated E. coli SQR and QFR (pH 7.8, 30°C)

<table>
<thead>
<tr>
<th>Quinone</th>
<th>Turnover no. (s⁻¹) catalyzed by:</th>
<th>SQR</th>
<th>QFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate-quinone reductase</td>
<td>Quinol-fumarate reductase</td>
<td>Quininate-quinone reductase</td>
<td>Quinol-fumarate reductase</td>
</tr>
<tr>
<td>Q₂</td>
<td>80</td>
<td>27</td>
<td>11</td>
</tr>
<tr>
<td>MQ₁</td>
<td>&lt;0.2</td>
<td>2</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Q₁H₂</td>
<td>4.8</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>MQ₂H₂</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* SQR was isolated from aerobically grown DW35 transformed with pSDH15.
* QFR was isolated from anaerobically grown DW35 transformed with pH3.

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 frdABC plasmids. As a control, DW35 transformed with pSDH15 (sdhCDAB driven from the PSDH 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 PPSRN-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 monocistronic plasmid with the PPSRN 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 b₅₅₆ 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 b₅₅₆ produced from the PFrD promoter with pFAS, 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 b₅₅₆ 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 pFAS or pFGS, 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).
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 oxidoreductase, normally a component of the aerobic respiratory chain, was expressed during anaerobic cell growth on glycerol-fumarate medium by using the PFRD 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

\[
\text{FIG. 2. Anaerobic cell growth. DW35 (}\text{frd} \text{sdh}C:\text{kan}) \text{ cells carrying pBR322 (Amp}\text{r}) (\bullet), pH3 (P}_{\text{frd}}\text{frdABCD in pBR322) (\text{○}), pH3-177 (P}_{\text{frd}}\text{frdABCD in pACYC177) (\triangle), pFGS (P}_{\text{frd}}\text{sdhC}^{\text{TAG}}\text{DAB in pBR322) (\times), pFGS-177 (P}_{\text{frd}}\text{sdhC}^{\text{TAG}}\text{DAB in pACYC177) (\ast), pFAS (P}_{\text{frd}}\text{sdhC}^{\text{ATG}}\text{DAB in pBR322) (\square), and pSDH15 (P}_{\text{frd}}\text{sdhCDAB in pBR322) (\text{△}) and strain MC4100 (wild type) with pBR322 (■) were grown on glycerol-fumarate medium supplemented with 0.025\% tryptone and 0.025\% yeast extract as described in Materials and Methods. OD600, optical density at 600 nm.}
\]

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Succinate-acceptor reductase activity (2 electron equivalents/min/mg of protein) (10(^{-6}))</th>
<th>Heme b(_{556}) (nmol/mg)</th>
<th>Turnover number(^{c}) (S(^{-1}))</th>
<th>Amplification level(^{d})</th>
<th>Doubling time (h)</th>
</tr>
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<td>frdABCD plasmid</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MC4100 (wild type)</td>
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<td>0.6</td>
<td>0.3</td>
<td>25</td>
<td>1</td>
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<tr>
<td>pH3-177</td>
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<tr>
<td>pFGS-177</td>
<td>14</td>
<td>—</td>
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<td>3.6</td>
<td>102</td>
</tr>
<tr>
<td>pSDH15</td>
<td>2.7</td>
<td>—</td>
<td>2.3</td>
<td>0.4</td>
<td>96</td>
</tr>
<tr>
<td>Aerobically grown cells(^{e}) (pSDH15)</td>
<td>10.5</td>
<td>9.4</td>
<td>1.5</td>
<td>100</td>
<td>ND(^{f})</td>
</tr>
</tbody>
</table>

\(^{a}\) 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.
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 pFGS 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, Pfrd-lacZ*, starting with the ATG codon, gave a fivefold-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 Pfrd-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 Pfrd-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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1984. Overproduction of fumarate reductase in *Escherichia coli* induces a 
Electron transfer from menaquinol to fumarate: Fumarate reductase anchor 
Identification of amino acid residues involved in catalytic activity with qui-
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