Partial Replacement of Succinate Dehydrogenase Function by Phage-
and Plasmid-specified Fumarate Reductase in Escherichia coli

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Phages capable of transducing succinate dehydrogenase mutants (sdh) of Escherichia coli were isolated from pools of artificially constructed recombinant lambda phages using a selective casein digest medium. These phages produced characteristically dense turbid plaques, and as prophages they increased the aerobic growth efficiencies of sdh mutants on complex media but were unable to promote growth with succinate as sole carbon and energy source (an essential feature of sdh+ strains). The phages were identified as fumarate reductase transducing phages (λfrdA) by the presence of a characteristic 4.9 kilobase pairs R.HindIII fragment of bacterial DNA, the expression of a polypeptide with a relative molecular mass of 72,000 (the frdA gene product) and by comparing their transducing activities with authentic λfrdA phages. In parallel studies a strain containing a ColE1:frd hybrid plasmid (pGS1 = pLC16.43) was characterized. Transfer of pGS1 to sdh mutants was accompanied by increased aerobic growth efficiencies on complex media and the ability to utilize succinate as sole carbon and energy source. It was concluded that fumarate reductase can replace succinate dehydrogenase but the extent of the reversal of the sdh lesion depends on frd gene dosage and the titration of the repressor which normally prevents aerobic synthesis of the reductase.

INTRODUCTION

Escherichia coli contains two membrane-bound flavoproteins catalysing the interconversion of fumarate and succinate, succinate dehydrogenase and fumarate reductase (Hirsch et al., 1963; Spencer & Guest, 1973). Succinate dehydrogenase functions in the tricarboxylic acid cycle and is induced aerobically but repressed under anaerobic conditions. By contrast, fumarate reductase is repressed in air or nitrate but derepressed anaerobically; it allows fumarate to serve as terminal acceptor in an energy-generating electron transport chain, which in turn enables E. coli to grow anaerobically with non-fermentable substrates. The strict division of function is evident from studies with succinate dehydrogenase (sdh) and fumarate reductase (frdA) mutants lacking one or both enzymes. For example, the presence of a functional frdA gene does not permit growth of sdh mutants on tricarboxylic acid cycle intermediates, nor does its absence affect the aerobic growth yields of sdh+ or sdh strains (Creaghan & Guest, 1978). Likewise, a functional sdh gene will not satisfy the need for fumarate reduction during anaerobic growth on glycerol plus fumarate. Sensitive tests, based on the anabolic roles of the enzymes, also confirm their functional non-interchangeability. Thus, the state of the sdh gene does not alter the succinate requirement of fumarate reductase plus isocitrate lyase double mutants during anaerobic growth on glucose, nor is the succinate auxotrophy of mutants lacking 2-oxoglutarate dehydrogenase affected by the state of the frdA gene (Creaghan & Guest, 1978).

However, it is possible that the distinct roles of the two flavoproteins are imposed by regulatory factors controlling the expression of the respective genes because direct tests for functional interchangeability of the actual gene products have not been made. Recently, it was shown that the aerobic repression of fumarate reductase could be lifted by multiple gene
duplication in the frdA region of the chromosome (Cole & Guest, 1979a, b) and by amplification of the lambda-cloned frdA gene following induction of appropriate λfrdA derivatives (Cole & Guest, 1978, 1980b). The mechanism probably involves titration of a specific repressor responsible for the aerobic repression. These studies did not establish whether the aerobically amplified frdA product could replace the physiological functions of succinate dehydrogenase. However, during a search for artificially constructed recombinant transducing phages (λsdh) containing the succinate dehydrogenase gene, phages capable of partially reversing the metabolic lesions of sdh mutants were isolated. These were shown to be λfrdA phages indicating that fumarate reductase can offset the metabolic consequences stemming from a deficiency in succinate dehydrogenase. Similar conclusions were reached in parallel studies with hybrid CoIE1 plasmids encoding fumarate reductase.

**METHODS**

*Phages and bacteria.* Pools of recombinant phages containing R.HindIII and R.EcoRI fragments of DNA from *E. coli* strain CR63 (supD) were constructed with the corresponding replacement vectors, λNM761 (srIal–2Δshn633 att–redA imm21 nin5 shnA5) and λNM816 (a derivative of λplac5 in which donor DNA is inserted left of the phage attachment site by displacing an R.EcoRI fragment that contains most of the lacZ gene, srIplac–srI2) of Wilson & Murray (1979), and using the methods of Bock et al. (1976). Other phages include two artificially constructed λfrdA phages, λG1F and λG40F (derivatives of λNM54, srIal–2Δatt–imm21 nin5 shnA5) which contain a 4-9 kb R.HindIII fragment encoding the fumarate reductase gene (Cole & Guest, 1980a), and phages λimm524, λimm521, λfrdA233, λfrdA, λimm524 and λvir for use as heteroimmune helper phages and in routine tests for immunity.

The strains of *E. coli* K12 included JRG653 (trpA9761 gal trpR ileR rpsL) and its derivatives JRG660 (sdh-18), JRG780 (frdA11), JRG1002 (sdh-0 gal+) and JRG1003 (frdA11, sdh-0 gal+) which have been described previously by Cole & Guest (1980a). Strain C600 (thr leu thi supE tonA) was used for routine phage propagation and assay and S159 (uvrA gal rpsL sup30) for the analysis of polypeptides after u.v.-irradiation. A copy of the Clarke & Carbon (1976) colony bank containing synthetic CoIE1 hybrid plasmids in strain JA200 (F–trpA851 thr leu lacY recA) was kindly provided by Dr R. A. Cooper.

*Media.* The minimal media used in growth tests and as selective media have been described previously (Creaghan & Guest, 1978; Cole & Guest, 1980a). The peptone broth contained (g l–1): Trypticase peptone (BBL), 10; NaCl, 5. The rich nutrient broth used for routine subculture and phage preparation was L broth minus glucose and contained (g l–1): Tryptone (Difco Bacto), 10; yeast extract (Bacto), 5; NaCl, 5; adjusted to pH 7.2. Media were solidified with agar (Bacto) at 15 or 10 g l–1 or, for peptone or water agar soft overlays, at 6.5 g l–1.

*Enzymology.* Cultures were grown in L broth (250 ml) at 37 °C with shaking, starting with an inoculum of 0.5% (v/v), and various unlabelled products by electrophoresis in agarose gels have been described previously (Cole & Guest, 1980a).

*Restriction analysis.* The methods for DNA digestion with restriction endonucleases and the analysis of the products by electrophoresis in agarose gels have been described previously (Cole & Guest, 1980a). Standards covering the range 0.59 to 23.7 kb, derived by digesting lambda (λcl857) DNA with R.HindIII, R.EcoRI, and R.HindIII plus R.EcoRI, were included in several tracks on all gels to provide a calibration for fragment size relative to mobility. The restriction endonucleases, R.HindIII and R.EcoRI, were purchased from Boehringer.

*Analysis of polypeptides formed after u.v.-irradiation and phage infection.* Cultures of *E. coli* S159 were grown at 37 °C in glucose minimal medium, resuspended in medium plus MgSO4 (20 mM) and irradiated with u.v. light (900 J m–2) according to the methods described by Cole & Guest (1980b). After irradiation, samples were infected at a multiplicity of 5 and pulsed with L-[13C]methionine [80 to 125 μCi ml–1 (3.0 to 4.6 MBq ml–1) The Radiochemical Centre, Amersham]. The radioactive proteins were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis using an acrylamide concentration of 10% (w/v), and various unlabelled protein standards were used in determining relative molecular masses (M,) according to Cole & Guest (1979b). The gels were stained and then dried for autoradiography.
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Fig. 1. Appearance of transduction plaques obtained with a succinate dehydrogenase mutant (JRG1002) as indicator strain (recipient) and the selective peptone medium. The transducing phage plaques (dense centre) are shown for λG84 (a) and λG85 (b) after one round of purification from a background of λNM761 recombinant and vector phages (faintly turbid centre).

RESULTS

Studies with artificially constructed λ transducing phages

Several independently constructed pools of recombinant lambda transducing phages containing R.HindIII and R.EcoRI fragments of E. coli DNA in the corresponding replacement vector, λNM761 and λNM816 (Wilson & Murray, 1979), were screened for their ability to transduce succinate dehydrogenase mutants (sdh). Two selective media were used. The minimal salts medium containing succinate as sole carbon and energy source was completely selective. The other medium contained peptone (pancreatic digest of casein) and permitted weak growth of the sdh mutants. It was reasoned that the less selective medium might provide better conditions for the development of transduction plaques comparable with those obtained with λptrp and λpbs phages and the corresponding auxotrophs. In the latter situation syntrophy between transductants and recipient bacteria is probably responsible for the formation of a transductant plaque. In the case of mutants with lesions in energy-generating functions, no such cross-feeding is possible but a weakly permissive medium could combine the conditions necessary for both plaque-formation and selectivity. No transducing activity was detected with the minimal medium but the recombinant phage lysates derived from λNM761 produced a significant proportion of very striking plaques with densely turbid centres on the peptone medium (Fig. 1). No comparable activity was detected with the recombinant phage pools constructed with the λNM816 vector and R.EcoRI fragments of bacterial DNA. Five phages of independent origin were selected for further study (G84, G85, G97, G98, G99).

Nutritional properties of lysogens and dilysogens. Lysogenic and dilysogenic (with λimm134) derivatives of a succinate dehydrogenase mutant (JRG1002) were prepared using two of the potential λsdh phages. None of these derivatives grew on succinate minimal agar nor was growth sustained in quantitative tests involving growth from large inocula in liquid medium (Fig. 2c). This indicated that the prophages were unable to complement the succinate dehydrogenase lesion of the mutants. However, the extent of growth of the lysogens on the peptone medium was significantly greater than that of the non-lysogenic mutant but not as...
Fig. 2. Effect of lysogenization by $\lambda$G84 and $\lambda$G85 on the growth of mutants lacking succinate dehydrogenase or fumarate reductase plus succinate dehydrogenase on (a) peptone broth, (b) L broth and (c) succinate minimal medium. Cultures (10 ml) were shaken at 37 °C in 250 ml Erlenmeyer flasks with optically matched side-arms and their absorbance was measured at 610 nm. The inocula consisted of washed suspensions of organisms grown for 16 h in L broth plus glucose (0-1%, w/v) for (a) and (b) or in glucose minimal medium for (c). Strains: ---, parental strain (JRG653); --, $sdh$ mutant (JRG1002); ---, $frd$ $sdh$ double mutant (JRG1003); mutants contained the prophages $\lambda$G84 and $\lambda$G85 as indicated.

Great as the level attained by the wild-type parental strain (Fig. 2a). The growth of a double mutant lacking fumarate reductase and succinate dehydrogenase activities (JRG1003) was poorer than that of the $sdh$ mutant but again the growth of the corresponding lysogens was equal to, or slightly better than, that of the single $sdh$ mutant (Fig. 2a). Using a rich tryptone/yeast extract medium (L broth) much greater yields were obtained with all the strains but the differences between mutants, lysogens and the wild-type were qualitatively similar to those on the weaker medium (Fig. 2b). No striking differences in growth rate were apparent with either medium nor were the results obtained in growth tests with dilysogenic derivatives any different from those described for the monolysogens. It would thus appear that the presence of the transducing prophages reverses in part, but not completely, the consequences of the $sdh$ lesion on growth yield.

Restriction analysis. The transducing phages were compared with the vector by digestion of the phage DNA with restriction endonucleases R.HindIII and R.EcoRI, separately and in combination, followed by analysis of the products by electrophoresis in agarose gels. The vector phage contains a replaceable R.HindIII fragment encoding the $supF$ gene. It is 8-8 kb and has a single R.EcoRI target 2-8 kb from one end (Fig. 3). A quantitative analysis of the restriction products of the five transducing phages indicated the presence of several types, differing in the number and orientation of cloned fragments. The results could be interpreted unambiguously and are summarized in Fig. 3. All of the transducing phages were characterized by the presence of a R.HindIII fragment of 4-9 kb containing a single R.EcoRI target, which generates double-digestion products of 3-33 and 1-49 kb. Phage G84 contained only the 4-9 kb fragment, whereas phages G85, G98 and G99 contained an 8-8 kb fragment, presumed to be the $supF$ segment of the vector, in tandem with the 4-9 kb fragment (Fig. 3). The orientations of the two cloned fragments relative to the phage genome were the same as in the vector (NM761) and G84. Phage G97 contained the same two R.HindIII fragments linked in the same relative orientation but inverted with respect to the phage genome (Fig. 3). The physical characteristics of the R.HindIII fragment common to
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Fig. 3. Physical maps based on restriction analyses of the phages used in this investigation. The relative positions of the R.HindIII (\(\triangleright\)) and R.EcoR1 (\(\blacksquare\)) targets are indicated and the sizes of the corresponding fragments are shown in kb. The segments of bacterial DNA are shown as boxes, shaded for the supF vector fragment, and marked with the sizes of the R.HindIII plus R.EcoR1 double-digestion products. For comparison the physical maps of two \(\lambda frdA\) phages, G1F and G40F, are included. These are derivatives of the insertion vector NM540, which differs from NM761 only with respect to the supF segment and the presence of an extra R.EcoR1 target in the right arm of the phage, because the \(\lambda att-red\) region is not deleted. The double-digestion vector products arising from hybrid fragments at the junctions between vector and cloned DNA and not shown above are 1.32 kb (left arm) and 5.25 kb or 4.21 kb (right arm, NM761 or NM540 derivatives, respectively).

The transducing phages are identical to those defined previously for several fumarate reductase transducing phages (Cole & Guest, 1980a). Physical maps of two \(\lambda frdA\) phages, G1F and G40F, representing both orientations of the cloned fragment are shown for comparison in Fig. 3. These differ only in the size of the right arm of the vector (NM540), which is not deleted in the \(\lambda att-red\) region. Thus it would appear that the partial reversal of the growth defect of the \(sdh\) mutants is probably mediated by a cloned \(frdA\) gene rather than the restoration of succinate dehydrogenase activity.

Functional characterization of the transducing phages. The prediction that the transducing phages producing large turbid plaques on the \(sdh\) mutants are really a class of \(\lambda frdA\) phages was confirmed by several observations. First, they were all active in transducing \(frdA\) mutants to a completely wild-type phenotype, at relatively high frequencies (>0.1 per plaque-forming unit), when tested by anaerobic selection on glycerol plus fumarate medium according to Cole & Guest (1980a). Furthermore, the corresponding lysogenic derivatives of \(frdA\) mutants, isolated without nutritional selection, all regained the ability to use fumarate as the terminal electron acceptor for growth. Secondly, an electrophoretic and autoradiographic analysis of the proteins formed after infecting u.v.-irradiated bacteria with a representative phage (G85) clearly indicated that it directed the synthesis of a product of \(M_r = 72000\) (Fig. 4). The same protein was produced by an authentic \(\lambda frdA\) phage (G40F) but not by the vector (NM540). This protein has been identified previously as a subunit of fumarate reductase, the \(frdA\) gene product (Spencer & Guest, 1974; Cole & Guest, 1979b, 1980b). Other differences between the labelling profiles for G85 and G40F were apparent but these are probably due to differences between the respective vectors and the presence of the \(supF\) fragment in G85. Finally, it was found that the \(\lambda frdA\) phages, G1F and G40F, produced densely centred plaques on peptone medium with \(sdh\) mutant indicators, that were
Fig. 4. Autoradiograph of L-[\textsuperscript{35}S]methionine-labelled polypeptides synthesized in u.v.-irradiated E. coli S159 after infection with the phages indicated and fractionation in a sodium dodecyl sulphate-polyacrylamide gel (10%). Labelling was from 5 to 23 min after infection. The relative molecular masses (M<sub>r</sub>) were estimated by comparison with unlabelled standards (indicated on the left) and the protein encoded by the bacterial <i>frdA</i> gene is shown at 72 000.
Table 1. Effect of plasmid pGS1 on the specific activities for succinate oxidation and fumarate reduction in E. coli

Ultrasonic extracts were prepared from exponential phase cultures of organisms grown aerobically in L broth and assayed as described in Methods. The specific activities are expressed as μmol substrate transformed (mg protein)^{-1} h^{-1}.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Succinate oxidation</th>
<th>Fumarate reduction</th>
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<tbody>
<tr>
<td>JA200</td>
<td>1.83</td>
<td>0.92</td>
</tr>
<tr>
<td>JA200(pGS1)</td>
<td>4.05</td>
<td>7.62</td>
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indistinguishable from those shown in Fig. 1. Moreover, lysogenization of sdh and sdh frd mutants (JRG1002 and JRG1003) by λfrdA prophages had essentially the same effects on growth yields in peptone broth and L broth as were originally observed with the selected phages (Fig. 2).

Lysogenic derivatives of strain JRG1003 (frdA sdh) containing λfrdA prophages (λG1F and λG40F) were used in an attempt to isolate mutant transducing phages containing an aerobically derepressed frdA gene capable of supporting growth on succinate minimal medium. Twelve independent succinate-utilizing derivatives of each lysogen were selected and lysates were prepared by u.v.-irradiation. However, transduction tests with sdh mutants (JRG660 and JRG1002) indicated that none of the lysates was capable of high-frequency transduction of the ability to grow on a selective succinate minimal medium (frequencies ≤ 4 \times 10^{-5} per plaque-forming unit) with or without fumarate (2 mM) added as a potential inducer of frdA gene expression. It would therefore appear that the succinate-utilizing derivatives of the lysogenic strains frdA^- sdh^- (λfrdA^+) must have arisen by mechanisms other than those affecting regulatory elements cloned with the frdA gene.

Studies with strains containing ColE1 hybrid plasmids

A copy of the colony bank of Clarke & Carbon (1976) carrying hybrid ColE1-DNA (E. coli) plasmids was screened for clones capable of transferring the succinate dehydrogenase or fumarate reductase functions to the corresponding mutants. Exponentially growing patch cultures of the donor strains were transferred to lawns of the mutant recipients spread on appropriate selective media containing streptomycin (200 μg ml^{-1}). Anaerobic selection on glycerol plus fumarate medium was used with the frd mutants (JRG780 and JRG1003) and succinate minimal medium and the peptone medium were used with the sdh mutants (JRG660, JRG1002 and JRG1003). One clone was found to transfer, by F-mediated conjugation, a hybrid ColE1 plasmid (pGS1 = pLC16.43), capable of restoring the wild-type Frd^+ and Sdh^+ nutritional phenotypes to all of the mutants. The plasmid was identified as a ColE1-frd hybrid by an enzymic analysis of the donor strain (Table 1). Compared with the host strain the specific activities for fumarate reduction and succinate oxidation were both amplified (8.3-fold and 2.2-fold, respectively). The greater effect on fumarate reduction clearly shows that the plasmid expresses fumarate reductase rather than succinate dehydrogenase. Restriction analysis with the isolated plasmid (pGS1) using R.HindIII and R.EcoRI indicated that it is very probably the same as another plasmid (pNU1) isolated from the same source by Edlund et al. (1979). Plasmid pNU1 carries the frd-linked amp operon specifying the chromosomal β-lactamase of E. coli, it includes the 4.9 kb R.HindIII fragment of DNA that is cloned in the λfrdA transducing phages, and has been extensively characterized. Quantitative growth tests with the conjugation products (Fig. 5) showed that the presence of the plasmid increases the growth efficiencies of the sdh and sdh frd mutants on both the weak peptone medium and the rich nutrient broth (L broth). The extents of growth were detectably greater than those observed with the corresponding mutants carrying λfrdA prophages, but more important was the ability of the plasmid to promote growth on
Fig. 5. Effect of the ColEl hybrid plasmid (pGS1) on the growth of mutants lacking succinate dehydrogenase or fumarate reductase plus succinate dehydrogenase on (a) peptone broth, (b) L broth and (c) succinate minimal medium. The growth conditions were as in the legend to Fig. 2. Strains: ——, parental strain (JRG653); ——, sdh mutant (JRG1002); ——, frd sdh double mutant (JRG1003); mutants contained the plasmid pGS1 as indicated.

succinate minimal medium (Fig. 5c). The growth was significant and sustained, albeit at a declining rate, over the period tested. This is in sharp contrast to the λfrdA lysogens which appeared to stop growing once their endogenous reserves had been exhausted. These results confirm that fumarate reductase can replace succinate dehydrogenase to a significant extent. The greater efficacy of the plasmid-encoded enzyme presumably stems from the multicopy nature of ColEl and the consequent increased capacity to titrate the repressors which normally prevent aerobic expression of the frd region.

DISCUSSION

The attempts to isolate λsdh transducing phages using weakly selective conditions, in order to encourage transduction plaque formation, clearly led to the selection of new classes of λfrdA phages. These phages and previously isolated λfrdA phages produced strikingly turbid-centred plaques with sdh mutant indicators on the peptone medium. The quantitative growth tests clearly showed that the growth deficiencies of sdh mutants could be offset by λfrdA prophages on nutrient broths but not on succinate minimal medium. Thus it would appear that the succinate dehydrogenase function can in part be replaced by fumarate reductase. The presence of the R.HindIII supF fragment in some of the λfrdA derivatives of λNM761 was not essential for the observed effects on the sdh mutants nor was the orientation of the cloned frdA fragment important.

It has been established previously that amplification of the frdA gene leads to aerobic derepression of fumarate reductase synthesis (Cole & Guest, 1978, 1979a, b, 1980b), presumably by titrating a specific repressor responsible for the aerobic repression. Thus it is possible that the effects of the λfrdA phages on plaque formation are dramatic because the frdA gene dosage of infected bacteria may be high and the centres of the plaques may become anaerobic. Both of these factors could augment frd expression by titrating an aerobic repressor and by providing conditions for anaerobic derepression, respectively. Nevertheless, one extra copy of the frdA gene (provided by a λfrdA prophage) was sufficient to generate a significant increase in aerobic growth efficiency.

Studies with the hybrid plasmid containing the frdA gene confirmed that fumarate reductase can replace succinate dehydrogenase. This plasmid, pGS1 (pLC16.43), selected from the ColE1–E. coli hybrid plasmid bank of Clarke & Carbon (1976), appeared to be the
same as the well-documented pNU1, isolated from the same source by virtue of encoding and amplifying the frdA-linked chromosomal β-lactamase gene (Edlund et al., 1979). The multicopy nature of the plasmid would appear to be responsible for its enhanced ability to reverse the effects of the sdh lesion, particularly with respect to promoting growth on succinate minimal medium. Thus a complete replacement of succinate dehydrogenase by fumarate reductase would appear possible when factors preventing the aerobic expression of the frd system are overcome.

The selectivity provided by succinate minimal medium with λfrdA lysogenic derivatives of sdh frdA mutants offered a very convenient method for selecting regulatory mutants which can express the frdA gene under non-inducing aerobic conditions. In the studies reported here none of the mutations appeared to be associated with the active λ-cloned frdA gene. Some could affect an unlinked repressor gene and this is being investigated. Recently, studies by Ruch et al. (1979) with frd-lac fusion strains have yielded three types of mutant altered in the expression of the hybrid operon. These include mutants which are constitutive both aerobically and anaerobically, and two classes which can be induced aerobically in the presence of fumarate.

No specific λsdh phages were detected in the recombinant phage pools containing R.HindIII or R.EcoRI fragments. This confirmed previous findings with vectors that have a lower capacity for cloned DNA (Cole & Guest, 1980a). Presumably the sdh gene must either be disrupted by these restriction endonucleases or it must be released in fragments too large to be cloned by the λ vectors.

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REFERENCES


