Identification of the formate dehydrogenases and genetic determinants of formate-dependent nitrite reduction by *Escherichia coli* K12

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(Received 25 January 1993; accepted 29 March 1993)

The formate dehydrogenases of *Escherichia coli* involved in electron transfer from formate to nitrite (Nrf activity: nitrite reduction by formate) have been identified. No previously undescribed selenoprotein was detected in bacteria grown under conditions optimal for the expression of Nrf activity. The Nrf activities of single mutants defective in either FdhN or FdhH were between 50 and 60 % that of the parental strain. A double mutant defective in both FdhN and FdhH retained less than 10 % of the activity of the FdhN⁺FdhH⁺ strain. No Nrf activity was detected in a triple mutant defective in FdhN, FdhH and FdhO or in the *selC* strain. It is concluded that all three of the known formate dehydrogenases of *E. coli* can contribute to the transfer of electrons from formate to the Nrf pathway. Mutants defective in Nrf activity and cytochrome *c*₅₅₂ synthesis were isolated by insertion mutagenesis or identified amongst strains received from the *E. coli* Genetic Stock Center. The mutations were located in at least three regions of the chromosome, including the 92 to 94 minute region which includes *fdhF*, the gene encoding FdhH required for formate hydrogenlyase activity. Fine structure mapping by P1 transduction established that the *nrf* mutations in the *fdhF* region were due to defects in three separable loci, all of which were independent of but close to *fdhF*. Clones were isolated from a cosmid library that complemented a deletion extending from *fdhF* into a region essential for Nrf activity. From these clones, plasmids were isolated that complemented only some of the Nrf[−] mutations in the 92 to 94 minute region, confirming the presence of different operons essential for Nrf activity and cytochrome *c*₅₅₂ synthesis in this region. Suggested reasons for this genetic complexity include the need for proteins involved in electron transfer from the various formate dehydrogenases to cytochrome *c*₅₅₂, for the attachment of the haem group to the apocytochrome and for cytochrome *c*₅₅₂ export into the periplasm.

Introduction

Anaerobic cultures of many enteric bacteria, including *Escherichia coli*, can use either formate or NADH to reduce nitrite rapidly to ammonia (Abou-Jaoudé *et al.*, 1979*a, b*; Cole, 1988; Page *et al.*, 1990). The two pathways are biochemically and genetically independent (Pope & Cole, 1982). Although the structures, synthesis and regulation of the components of the NADH-dependent pathway have been thoroughly characterized both biochemically and genetically (Cole, 1988; Harborne *et al.*, 1992), far less is known about the less active, formate-dependent pathway (Nrf: nitrite reduction by formate).

Fujita & Sato (1966*a, b*; 1967) were the first to associate cytochrome *c*₅₅₂ with nitrite reduction to

ammonia by *E. coli*. Abou-Jaoudé *et al.* (1979*a, b*) established that formate was the electron donor for a membrane-associated electron transfer chain to nitrite and Motteram *et al.* (1981) demonstrated that a membrane potential was generated during Nrf activity. Twenty years after its first published purification and characterization by Fujita (1966), Kajie & Anraku (1986) repurified cytochrome *c*₅₅₂ and reported it to be a 69 kDa polypeptide with six associated haem groups. The aims of the experiments described here were to identify the dehydrogenases which transfer electrons from formate to the cytochrome and to locate *E. coli* chromosomal genes essential for Nrf activity.

Methods

Strains used and their source. All strains used in this work are listed in Table 1 together with details of their source or method of construction.

Media and growth conditions. To determine rates of nitrite reduction by formate or glucose, bacteria were grown in unshaken 1.0 or 2.0 litre conical flasks filled with the minimal salts (MS) medium described by

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Table 1. *Strains used and their source*

Strain	Relevant genotype or phenotype	Reference or source
CGSC 4260	JC 1553 F' 112	E. coli Genetic Stock Center, Yale Univ., New Haven, CT, USA
CGSC 4312	HFr P4X <i>metB</i>	
CGSC 4432	<i>purA rpsL</i>	
CGSC 4517	<i>pyrB</i>	
CGSC 4926	HFr Cavalli <i>metB1 melA7</i>	
CGSC 10235	HFrH	Rossmann <i>et al.</i> (1991)
FM460	MC4100 $\Delta selC::Kan^R$	
FM932	$\Delta fdhF mel::Tn10$	
JCB351 to JCB379	$\Delta nirB nrf-351$ to 379	This work: Nrf ⁻ temperature-stabilized Mud1 insertions in JCB387
JCB387	E. coli RV $\Delta nirB$	Transduce JCB303 (Griffiths & Cole, 1987) to Nar ⁺ with P1 from E. coli RV
JCB601 to JCB606	$\Delta nirB nrf-601$ to 606	This work: Nrf ⁻ λ placMu insertion mutants of JCB387
JCB3406	M9 $\Delta nirB nrf^+$	Transduce M9 <i>cysG</i> to Cys ⁺ with JCB387-P1
JCB3513	CGSC 4432 <i>nrf-351</i>	Transduce CGSC4432 to Amp ^R with CGSC4432-P1
JCB3876	JCB387 <i>fdhF</i>	Transduce JCB387 to Amp ^R with FM909-P1
JCB3877	JCB387 <i>fdnG</i>	Transduce JCB387 to Kan ^R with VJS1224-P1
JCB3878	JCB387 $\Delta(nrf-fdhF)$	Transduce JCB387 to Tet ^R with FM932-P1
JCB3880	JCB387 <i>fdnG fdhF</i> (Amp ^R)	Transduce JCB3877 to Amp ^R with P1 from a temperature-stabilized derivative of M9
JCB3881	JCB387 $\Delta sel::Kan^R$	Transduce JCB387 to Kan ^R with FM460-P1
JCB3882	JCB387 <i>fdnG fdhF fdo</i>	Transduce CGSC 4432 to Tet ^R with 5278-P1; then to Kan ^R with JCB602–JCB606-P1
JCB6023	CGSC 4432 <i>narL::Tn10 nrf-602</i>	
JCB6033	CGSC 4432 <i>narL::Tn10 nrf-603</i>	
JCB6043	CGSC 4432 <i>narL::Tn10 nrf-604</i>	
JCB6053	CGSC 4432 <i>narL::Tn10 nrf-605</i>	
JCB6063	CGSC 4432 <i>narL::Tn10 nrf-606</i>	Transduce JCB387 with FM932-P1
JCB6061	JCB387 $\Delta(nrf-fdhF) mel::Tn10$	
JCB6069	JCB606 <i>mel::Tn10</i>	Transduce JCB606 to Tet ^R with FM932-P1
JM712	<i>pro his trp</i> Δlac	M. Jones-Mortimer
MA50	<i>fdo::MudA</i> Kan ^R	M.-A. Mandrand (unpublished)
M9	<i>fdhF</i>	Pecher <i>et al.</i> (1983)
M9 <i>cysG</i>		Transduce M9 to Kan ^R with JCB322-P1
MC4100		Casadaban & Cohen (1979)
MC4100 <i>cysG</i>		Transduce MC4100 to Kan ^R with JCB322-P1 (Griffiths & Cole, 1987)
VJS1224	<i>fdnG::MudIII</i> 1734 (Kan ^R)	Berg & Stewart (1990)

Pope & Cole (1982) supplemented with half-strength nutrient broth and 10 μ M-selenate and -molybdate (Page *et al.*, 1990). The main carbon and energy source was either 0.4% (w/v) glucose or 0.4% (w/v) glycerol + 40 mM-fumarate, as noted.

Inocula for anaerobic batch cultures were grown aerobically to late exponential phase in 20 ml of Lennox broth (LB). Stock cultures were maintained at -20 °C in LB supplemented with 20% (v/v) glycerol. Working cultures were maintained for up to 8 weeks on nutrient or Lennox agar at 4 °C.

Insertion mutagenesis. E. coli strain JCB387 was mutagenized with Mud1Aplac as described previously (Griffiths & Cole, 1987). Ampicillin-resistant colonies were purified on nutrient agar (NA) supplemented with 50 μ g ampicillin (Amp) ml⁻¹ and tested for Nrf activity as described below. Mutagenesis of an overnight culture of JCB387 which had been grown in tryptone broth with λ placMu9 was essentially as described by Bremer *et al.* (1984) except that bacteria were suspended in 10 mM-MgCl₂ before mutagenesis. In each experiment, two different infection mixes were set up: one contained 4 μ l of λ placMu9 and 40 μ l of helper λ pMu507 (Bremer *et al.*, 1985); 10 times as much of each phage were added to the second infection mix. Each transduction mixture was diluted to 10⁻⁴, 10⁻⁵ and 10⁻⁶ and 100 μ l aliquots were plated onto NA + Amp.

Rates of nitrite reduction by washed bacteria and cytochrome *c*₅₅₂ concentrations. The rates of nitrite reduction by formate (Nrf activity) or glucose were determined exactly as described by Pope & Cole (1984). Cytochrome *c*₅₅₂ concentrations in either the high speed supernatant fraction from bacteria broken in the French press (Pope & Cole, 1984) or from the periplasm fraction released by incubating bacterial suspensions with chloroform (Ames *et al.*, 1984) were determined spectroscopically as described by Pope & Cole (1984). Control experiments revealed that not all of the cytochrome *c*₅₅₂ was released quantitatively by the chloroform procedure, but as the ratio of cytochrome released to total protein were almost identical in both types of preparation, the two methods were used interchangeably. The two types of preparation are distinguished in the Results section by referring to them as soluble proteins (released by the French press) and periplasmic proteins (released by chloroform treatment).

Determination of phenotypes of Nrf⁻ mutants, exconjugants and transductants. Recombinants from conjugation or transduction experiments were purified by replating them onto the original selective agar and screened for unselected markers. Formate hydrogenlyase activity (Fhl⁺) was detected by growing bacteria overnight in 5 ml of LB + 0.8% (w/v) glucose in narrow test-tubes containing an inverted Durham tube. Strains that were Fhl⁺ (and therefore *fdhF*⁺)

accumulated H_2 in the Durham tube. Mutants defective in the *fnr* gene failed to form colonies during anaerobic growth on minimal agar with 0.4% (w/v) glycerol and 40 mM-fumarate as the carbon and energy source. To score the nitrate reductase (Nar) phenotype, bacteria were grown anaerobically in LB supplemented with 0.4% (w/v) glucose and 20 mM-nitrate, sedimented by centrifugation, resuspended in 50 mM-phosphate, pH 7.4, to a density of approximately 1 mg dry wt ml⁻¹ and incubated with 40 mM-formate and 20 mM-nitrate. In contrast to Nar⁺ strains, which had accumulated nitrite within 2 min, Nar⁻ strains were negative (or only weakly positive) for nitrite after 10 min at 30 °C. Chlorate-resistant (Mol⁻; formerly Chl^R) strains formed colonies during overnight anaerobic growth at 37 °C on LB supplemented with 0.4% (w/v) glucose and 10 mM-potassium chlorate. Mel⁺ strains formed colonies on minimal agar + 0.2% (w/v) melibiose after 2 d at 30 °C.

Many attempts to devise a selective medium for Nrf⁺ strains that were defective for the more active Nir pathway were unsuccessful. The Nrf phenotype was therefore scored by inoculating a test-tube containing 1 ml of LB + glucose + 1 mM-nitrite with the strain to be tested. After overnight growth without aeration, 3 ml of MS + glucose was added. After a further 3 h at 37 °C without aeration (or when visible growth had occurred), 50 µl samples of an isogenic Nrf⁺ control culture were spot-tested for nitrite. When no nitrite was present in the Nrf⁺ control, 0.5 ml 1% (w/v) sulphanilamide in 1 M-HCl and 0.5 ml 0.02% *N*-1-naphthylethylenediamine dihydrochloride were added to the remaining cultures. The formation of an intense pink colour after 30 s indicated the presence of nitrite and a Nrf⁻ phenotype. The original Nrf⁻ mutants were originally detected by a slightly modified procedure in which 1 ml of LB overnight cultures of bacteria from individual colonies were supplemented with 3 ml of MS + glucose and 1 mM-nitrite.

Although the Nrf pathway is partially repressed during growth in LB + glucose and derepressed in MS supplemented with glycerol + fumarate, attempts to improve the discrimination between Nrf⁺ and Nrf⁻ strains under the latter conditions were only partially successful because many Nrf⁺ strains grow poorly and are therefore falsely scored as Nrf⁻. The Nir phenotype was determined in a similar procedure except that individual colonies were grown aerobically overnight in 1 ml of LB and supplemented with 3 ml of MS + 0.4% glucose and 2.5 mM-nitrite.

Recombinant DNA techniques. Small-scale DNA preparations, restriction digests, agarose and polyacrylamide gel electrophoresis, the isolation of DNA fragments, the preparation and transformation of competent bacteria and other routine DNA manipulations were as described by Maniatis *et al.* (1982). Chromosomal DNA for cloning into the EMBL cos4 vector was prepared from a 20 ml overnight LB culture as described by Chesney *et al.* (1979) and size-fractionated by caesium chloride gradient centrifugation. High M_r DNA was partially digested with *Sau*3A to generate fragments larger than 35 kilobases (kb), as judged by electrophoresis on a 0.2% agarose gel. These fragments were size-fractionated by density gradient centrifugation; fractions with fragments in the range 35 to 48 kb, as judged by agarose gel electrophoresis, were dephosphorylated with calf alkaline phosphatase, extracted with phenol and precipitated with ethanol. The cos4 vector was restricted to completion with *Pvu*II, dephosphorylated, extracted twice with phenol/chloroform, ethanol precipitated and restricted with *Bam*HI to inactivate the tetracycline resistance gene and generate the two cosmid arms. After further phenol extraction and ethanol precipitation, 1.5 µg of the cosmid arms DNA was ligated with 3 µg of the purified, size-fractionated chromosomal DNA. After 16 h at 14 °C, 4 µl aliquots of the ligation mixture were packaged according to the Stratagene GIGAPACK Gold packaging procedure. The cosmid library was transformed into competent JCB3878 and Amp^R transformants were selected on nutrient agar + 50 µg Amp ml⁻¹.

Sub-cloning of *nrf* DNA into a low copy number plasmid vector. DNA prepared from two of the cosmids that transformed JCB3878 to Nrf⁺ was partially digested with *Sau*3A and ligated to pLG339 DNA (Stoker *et al.*, 1982) that had been restricted with *Bam*HI and dephosphorylated. Competent JCB3878 bacteria were transformed to kanamycin resistance (Kan^R) with the ligation mix. The 200 transformants were screened for Nrf⁺ clones; plasmid DNA was isolated from six of the Nrf⁺ clones and the partial restriction maps of three of the smallest clones were determined. The sub-clone with the smallest DNA fragment, pLD5, that consistently transformed JCB3878 to Nrf⁺, was used for the majority of the subsequent experiments.

The only selectable marker on pLD5 was Kan^R. To generate a comparable plasmid suitable for transformation into the Kan^R Nrf⁻ mutants, the 9 kb *Eco*RI/*Sal*I fragment from pLD5, which includes all of the *E. coli* chromosomal DNA and about 300 bases of the *tet* gene from pLG339, was purified from an agarose gel and ligated into the purified 3.7 kb *Eco*RI/*Sal*I fragment of pBR322. Competent JCB3878 was transformed with the ligation mix and Amp^R transformants were selected on Lennox agar + 50 µg Amp ml⁻¹.

Labelling of proteins with ⁷⁵selenium. Selenate was omitted from media used for these experiments. Bacteria were first cultured without aeration in 1 ml of either MS, glycerol and fumarate or in LB + 0.4% glucose, both of which were also supplemented with 0.25 µM-sodium selenite. Some cultures also contained either 20 mM-nitrate or 2.5 mM-nitrite. From these inocula, 10 µl of the LB cultures or 200 µl of the MS cultures were transferred to two test-tubes containing 3 ml of identical medium. One culture of each type was supplemented with 0.25 µM unlabelled selenite; to the other was added 0.25 µM [⁷⁵Se]selenite [specific activity 1.328 mCi µmol⁻¹ (49.14 MBq µmol⁻¹)]. After 4 h growth at 37 °C, the OD₆₅₀ of the unlabelled culture was measured as an estimate of the density of the ⁷⁵Se-labelled culture. Samples of the latter (1 ml) were pelleted by centrifugation and stored at -20 °C.

SDS-PAGE and autoradiography. Bacterial pellets were resuspended in sample buffer to a density of 25 mg bacterial mass ml⁻¹, heated to 100 °C for 10 min, cooled on ice and 20 µl samples were immediately loaded onto a 15% (w/v) SDS-PAGE gel. After electrophoresis for 17 h at 70 V, the resolving gel was transferred to Whatmann 3MM chromatography paper, dried under vacuum at 80 °C and autoradiographed.

Results

Transfer of electrons from formate into the Nrf pathway

Three formate dehydrogenases have been characterized in *E. coli*, each of which is a selenoprotein associated with a separate electron transfer pathway (Sawers *et al.*, 1991). In previous ⁷⁵Se labelling experiments, bacteria had been grown in rich media or in the presence of nitrate, conditions in which Nrf activity is at least partially repressed (Cox *et al.*, 1981; Pecher *et al.*, 1985; Leinfelder *et al.*, 1988; Sawers *et al.*, 1991). As it was possible that a fourth selenoprotein specific to the Nrf pathway had been undetected, selenoproteins synthesized during anaerobic growth under conditions optimal for Nrf expression were compared with those in bacteria grown in rich, glucose-containing medium. Strain JCB387 (FdhF⁺ FdhN⁺ FdhO⁺) was grown anaerobically in the presence of [⁷⁵Se]selenite in both LB + glucose with or without 20 mM-nitrate and in minimal salts supplemented with glycerol, fumarate and

Fig. 1. Selenoproteins synthesized by a double mutant of *E. coli* defective in both of the major formate dehydrogenases and by the parental strain after growth in different media. The FdhN⁻FdhF⁻ mutant, strain JCB3880, and its parental strain JCB387 were grown anaerobically in the presence of [⁷⁵Se]selenite in either Lennox broth (LB) or in minimal medium (MS) with various supplements, as listed. Proteins in 20 µl of the cell lysates were separated by SDS-PAGE and selenoproteins were detected by autoradiography. Tracks 1 to 4, JCB387; tracks 5 to 8, strain JCB3880. The growth media were LB + glucose (tracks 1 and 5); LB + glucose and 20 mM-nitrate (tracks 2 and 6); MS + 0.4% (w/v) glycerol + 40 mM-fumarate (tracks 3 and 7); MS + glycerol, fumarate and 2.5 mM-nitrite (tracks 4 and 8).

either 2.5 mM-nitrite or 20 mM-nitrate. Radiolabelled proteins were separated by SDS-PAGE and detected by autoradiography (Fig. 1). The 80 kDa product of the *fdhF* gene was the major selenoprotein detected in bacteria grown in LB + glucose. Synthesis of this polypeptide was repressed by nitrate, but the 110 kDa FdnG selenoprotein was induced (Fig. 1). The *fdnG* product was also induced during growth in minimal medium supplemented with nitrate, and small quantities of both the FdhF and FdnG polypeptides were detected in bacteria grown in nitrite-supplemented minimal medium.

As FdnN and FdhO are both 110 kDa polypeptides, the poorly expressed FdhO could not be seen in the above experiments. They were therefore repeated using a double mutant, JCB3880, which is defective in both the *fdhF* and *fdnG* genes. No clear selenoprotein band was detected in this double mutant after growth in LB + glucose due to the lack of a functional *fdhF* gene.

The addition of nitrate to LB induced a very low level of expression of the 110 kDa FdhO polypeptide (Fig. 1). The poorly expressed FdhO polypeptide was also the only selenopeptide detected in bacteria grown in minimal medium with glycerol, fumarate and nitrite. These results established that no novel selenoprotein is synthesized when bacteria are grown under conditions optimal for Nrf expression.

Contribution of each of the three known formate dehydrogenases to nitrite reduction by formate

Isogenic strains lacking one or both of the major nitrite reductases as well as a triple mutant lacking all three enzymes were constructed. The cytochrome content and Nrf activities of these strains were compared with those of the parental strain (Table 2). As these strains grew very poorly in minimal medium with glycerol and fumarate as the main carbon and energy source, bacteria were grown in minimal salts supplemented with 5% (v/v) LB, glucose and nitrite.

Both of the single mutants were Nrf⁺, but the rates of nitrite reduction by washed bacteria were significantly lower than that of the parental strain. Only a very low rate was detected in the FdhF⁻FdhN⁻ double mutant, and this was undetectable in the triple mutant. Although these data strongly imply that all three formate dehydrogenases can transfer electrons from formate into the Nrf pathway, there was no parallel loss of glucose-dependent rates of nitrite reduction (Table 2), indicating that the strains were able to use one or more electron donors other than formate or NADH to reduce nitrite.

A mutant incapable of incorporating selenocysteine into polypeptides was constructed by transducing the Nir⁻Nrf⁺ strain JCB387 to *selC*:Kan^R with bacteriophage P1 propagated on strain FM460 (Sawers *et al.*, 1991). The resulting strain, JCB3881, was devoid of formate-dependent nitrite reduction, confirming the original report by Abou-Jaoudé *et al.* (1979b) that a selenoprotein is essential for the transfer of electrons from formate to cytochrome *c*₅₅₂. Similar quantities of cytochrome *c*₅₅₂ [typically in the range 120 to 200 pmol (mg soluble protein)⁻¹ but higher in the triple mutant] were synthesized by all of the strains used in this study (Table 2).

Isolation of mutants defective in formate-dependent nitrite reduction

Strains defective in Nrf activity were from four sources. The first strains, numbered JCB351 to 379, were generated by insertion mutagenesis of the prototrophic, Nir⁻, *E. coli* strain JCB387 by the temperature-sensitive operon fusion phage, Mud1Aplac. Subsequently, further

Table 2. Contribution of the three different formate dehydrogenases to formate-dependent nitrite reduction

Strain	Relevant phenotype	Nitrite reduction by:*		Cytochrome c_{552} concn†
		glucose	formate	
JCB387	FdhN ⁺ FdhH ⁺ FdhO ⁺	0.71	1.05	120
		0.93	1.02	176
JCB3876	FdhN ⁺ FdhH ⁻ FdhO ⁺	0.74	0.58	201
		0.70	0.44	128
JCB3877	FdhN ⁻ FdhH ⁺ FdhO ⁺	0.75	0.59	139
		0.55	0.45	120
JCB3880	FdhN ⁻ FdhH ⁻ FdhO ⁺	0.44	0.10	176
		0.46	0.12	360
JCB3881	Sel ⁻ (FdhN ⁻ FdhH ⁻ FdhO ⁻)	0.66	< 0.01	160
		0.43	< 0.01	206
JCB3882	FdhN ⁻ FdhH ⁻ FdhO ⁻	0.41	< 0.01	389
		0.40	< 0.01	642

* Rates of nitrite reduction are expressed as $\mu\text{mol nitrite reduced h}^{-1}$ (mg bacterial dry mass)⁻¹.

† Cytochrome concentrations are nmol cytochrome c_{552} (g soluble protein)⁻¹.

Table 3. Characterization of λplacMu insertion mutants of *E. coli* JCB387 defective in formate-dependent nitrite reduction

Colonies of each insertion mutant were also tested for ability to reduce phenazonium methosulphate or benzyl viologen (FdhN or FdhH phenotype, respectively), but the results were too ambiguous for phenotypes to be assigned with any certainty.

Phenotype determined	Group 1	Group 2	Group 3	Group 4	Group 5
Nrf activity	—	—	—	—	—
Fnr test: growth on glycerol + fumarate	—	+	—	+	+
Nitrate reduction	—	+	—	+	+
Formate hydrogenlyase: H ₂ production from glucose	—	—	—	+	+
Chlorate resistance	—	—	+	—	—
Cytochrome c_{552}	—	ND	ND	+	—

ND, Not determined.

insertion mutants were generated in the same host using the protein fusion phage $\lambda\text{placMu9}$. Attempts to isolate mutants defective in periplasmic proteins using the *TnphoA* vector (Manoil & Beckwith, 1985) yielded disappointing results. Although many Amp^R mutants were isolated, approximately 2% of which gave blue colonies on X-P agar, only one Nrf⁻ mutant was found amongst over 6000 screened. In contrast, the most prolific source of Nrf⁻ strains was the *E. coli* Genetic Stock Center: about half of the auxotrophic strains requested for mapping experiments were subsequently found to be Nrf⁻ and were therefore unsuitable for the purpose for which they were obtained.

Antibiotic-resistant transposon insertion strains were first tested individually for Nrf activity by growing them in 10 ml of LB + glucose supplemented with 1.0 mM-nitrite in test-tubes. Cultures which had failed to reduce the nitrite after 6 h of anaerobic growth were retested

twice in the same way and then the Mol, Nar, Fnr and Fhl phenotypes were determined. Over 90% of the initial Nrf⁻ isolates were also defective for other activities (Table 3). Many were resistant to chlorate during anaerobic growth (due to mutations in a *mol* gene required for molybdenum cofactor synthesis; Group 3, Table 3), unable to reduce nitrate rapidly (mutants defective in haem or molybdenum cofactor synthesis or in the *fnr* genes; Groups 1 to 3), or failed to produce H₂ gas from glucose due to insertions in *fnr* or one of the *sel* genes (Groups 1 and 3; Table 3). These strains were discarded. Selected Nrf⁻ strains which were apparently wild-type for all of the above activities (Groups 4 and 5, Table 3) were grown in 2 litre anaerobic cultures, harvested, and the rates of glucose- and formate-dependent nitrite reduction by washed bacterial suspensions were determined (Table 4). The bacteria were broken in the French press and the concentration of

Table 4. Glucose- and formate-dependent rates of nitrite reduction and cytochrome c_{552} content of λ placmu-induced *Nrf* mutants unaffected in other aspects of anaerobic metabolism

Nrf mutant tested	Nitrite reduction by:*		Cytochrome c_{552} concn†
	glucose	formate	
JCB 323 (parental strain)	8.9	10.9	235
JCB351	1.1	< 0.1	< 10
JCB352	2.1	< 0.1	tr.
JCB353	2.5	< 0.1	tr.
JCB354	< 0.1	< 0.1	< 10
JCB355	< 0.1	< 0.1	< 10
JCB362	0.6	< 0.1	tr.
JCB379	0.5	0.3	tr.

* Nitrite reduced min^{-1} (mg bacterial dry mass) $^{-1}$.

† pmol cytochrome (mg soluble protein) $^{-1}$.

tr., Too little to quantify, but a very small spectral peak clearly visible: see fig. 2(b) for an example (JCB352).

cytochrome c_{552} in the soluble fraction after high speed centrifugation was determined (Table 4).

Virtually no *Nrf* activity was detected with suspensions of any of the mutants tested and only very low residual rates of nitrite reduction by glucose were found. All of the mutants were deficient in c -type cytochromes, but some variations were noticed. For example, although the concentration of cytochrome c_{552} in strain JCB352 was less than 10% of the parental strain, a clear peak of residual cytochrome was consistently seen in absorption spectra (Fig. 2). In contrast, no soluble cytochrome was ever detected in cultures of some of the other mutants such as JCB351 or JCB606. This was the first indication that the various *Nrf* $^{-}$ mutants were defective at different loci.

The insertion mutations of strains which had repeatedly been shown to be defective in *Nrf* activity by all of the above criteria were transferred by P1-mediated transduction to the *Nir* $^{-}$ *Nrf* $^{+}$ strain, JCB387, for further analysis. Some of the mutations were also transferred to a variety of HFr strains with different origins of chromosomal transfer, or to *F* $^{-}$ auxotrophic strains suitable for genetic mapping experiments. In most cases, the *nir* operon was first deleted from these strains to enable the *Nrf* phenotype of recombinants from subsequent experiments to be scored. In this way it was shown that strains MC4100, one culture (but not another!) of the HFrH strain CGSC 10235, CGSC 4517 and HFr P4X were all *Nrf* $^{-}$.

Chromosomal location of some of the mutations responsible for the Nrf $^{-}$ phenotypes

In preliminary interrupted mating experiments with

Fig. 2. Absorption spectra of soluble cytochromes from two *Nrf* $^{-}$ mutants and the parental strain, JCB387. Bacteria were grown anaerobically in 2 litre flasks filled with half-strength nutrient broth in minimal salts supplemented with 0.4% glucose and 2.5 mM-nitrite. Bacteria were harvested by centrifugation, resuspended in 50 mM-phosphate buffer, pH 7.4, and broken in a French press. Soluble proteins were prepared by centrifugation for 1 h at 150 000 g. One sample was reduced with a few grains of sodium dithionite; the reference sample was oxidized with a small crystal of potassium ferricyanide. The difference spectrum (reduced minus oxidized samples) was recorded. Protein concentrations were 13.5 mg ml $^{-1}$ (parental strain JCB387; spectrum a), 13.3 mg ml $^{-1}$ (JCB352; spectrum b) and 4.6 mg ml $^{-1}$ (JCB606; spectrum c).

antibiotic-resistant *Nrf* $^{-}$ HFr strains as the donor, many *Mel* $^{+}$ *Nrf* $^{-}$ recombinants were isolated, suggesting that some of the *Nrf* mutations were located in the 90 to 100 minute region of the *E. coli* chromosome. To investigate this further, a *Nir* $^{-}$ derivative of the HFr Cavalli strain, CGSC 4926, with defects in both *mel* and *metB* genes, was the donor for uninterrupted mating experiments with various *Pur* $^{-}$ *Nrf* $^{-}$ *narL*::Tn10 recipients: *Ade* $^{+}$ *Tet* R recombinants were selected (Table 5). Similar results were obtained in further conjugation

Table 5. Analysis of recombinants obtained from conjugating an HFr Cavalli derivative, CGSC 4926, with adenine-auxotrophic *Nrf*⁻ mutants

The donor strain was CGSC 4926 with the relevant genotype *pur*⁺ *mel* *metB*.*narL*⁺; the recipients were *narL*:Tn10 *Nrf*⁻ λ placMu9 derivatives of CGSC 4432. After 1 h of uninterrupted mating in Lennox broth at 37 °C, *Pur*⁺ *Tet*^R recombinants were selected and, after purification, analysed for the inheritance of unselected markers.

Recombinant class	Nrf ⁻ mutation in recipient				
	<i>nrf</i> -602	<i>nrf</i> -603	<i>nrf</i> -604	<i>nrf</i> -605	<i>nrf</i> -606
Ade ⁺ Met ⁺ Mel ⁺ Kan ^R	27	19	28	23	24
Mel ⁺ Kan ^S	0	0	1	9	5
Mel ⁻ Kan ^R	51	53	2	24	2
Mel ⁻ Kan ^S	0	1	39	21	35
Ade ⁺ Met ⁻ Mel ⁺ Kan ^R	0	0	0	0	0
Mel ⁺ Kan ^S	0	0	0	0	0
Mel ⁻ Kan ^R	6	9	0	5	0
Mel ⁻ Kan ^S	0	0	15	5	12

experiments with *Nrf*⁻ Amp^R derivatives of CGSC 4432 (not shown). Analysis of the recombinants revealed that the antibiotic resistance determinants associated with the *Nrf* insertion mutations were located in at least three separate segments of the chromosome.

The significant numbers of antibiotic-sensitive, Mel⁻ recombinants in matings with recipients carrying the mutations from *Nrf*⁻ strains JCB351, 362, 379, 604 and 606 indicated that the defective *nrf* genes in these strains were located in the 90 to 95 minute region of the chromosome (Fig. 3a). The mutations in this group were transferred to an F⁻ *metB* strain, and F'112, which includes chromosomal DNA from the 88 to 98 minute region of the *E. coli* chromosome, was transferred into these mutants by conjugation with selection for Met⁺. The exconjugants were all *Nrf*⁺.

In contrast to the majority of mutants screened, all of the recombinants from matings with recipients with mutations from strains JCB602 and 603 were Kan^R, consistent with the *nrf* mutations being located in the 40 to 80 minute segment of the chromosome. Conversely, the pattern of inheritance of unselected markers in matings with a recipient carrying the mutation from strain JCB605 implied that the defective *nrf* gene was located between *purA* at minute 95 and the origin of transfer at minute 13 (Table 5; Fig. 3a).

Co-transduction of the *Nrf*⁻ phenotype of *E. coli* strain FM932 with *fdhF*

Some of the *Nrf* mutations, which in conjugation experiments appeared to be linked to the *mel* operon, were mapped more precisely by transduction using *mel*

Fig. 3. Region of the *E. coli* chromosome in which various mutations conferring a *Nrf*⁻ phenotype were located. (a) The arrowhead indicates the origin of chromosomal transfer by the HFr strain CGSC 4926. The positions of relevant genes in the 88 to 98 region of the chromosome are also indicated. (b) The physical map of the *fdhF* to *mel* region, as reported by Kohara *et al.* (1987). (c) Partial restriction maps of three plasmids which complement several of the *nrf* mutations located in the 92 minute region of the chromosome.

and *fdhF* mutations as the reference markers. First, the temperature-stabilized *fdhF* deletion strain FM932 was transduced to *cysG*:Tn5 to inactivate the Nir pathway and then checked for *Nrf* activity. Unexpectedly, all of the transductants were both Nir⁻ and *Nrf*⁻. Strain FM932 had been constructed from MC4100 in three steps: insertional inactivation of *fdhF* with Mud1Aplac to give the temperature-sensitive *fdhF* mutant, M9; temperature stabilization, which resulted in the deletion of *fdhF* and an unknown length of adjacent DNA, and transduction to *mel*:Tn10 (Pecher *et al.*, 1985). Although the *Nrf*⁻ phenotype of FM932 was the first indication (shown above to be incorrect) that *fdhF* might

Table 6. *Formate-dependent nitrite reduction and cytochrome c_{552} synthesis by E. coli strain MC4100 and its derivatives*

Bacteria were grown anaerobically in unshaken, deep standing cultures in NCM supplemented with 0.4% glycerol, 40 mM-fumarate and 2.5 mM-nitrite. The chloroform method was used to extract periplasmic cytochrome c_{552} in these experiments.

Strain	Nitrite reduction by formate*	Cytochrome c_{552} concn†
MC4100 <i>nirB</i>	< 0.1	106
MC4100 <i>cysG</i> : :Tn5	< 0.1	88
MC4100 <i>cysG</i> : :Tn5 Nrf ⁺	22.2	422
M9 <i>cysG</i> : :Tn5	< 0.1	94
M9 <i>cysG</i> : :Tn5 Nrf ⁺	29.4	401
JCB387 <i>nirB</i>	17.0	389

* nmol nitrite reduced min⁻¹ (mg bacterial dry mass)⁻¹.

† pmol cytochrome (mg soluble protein)⁻¹.

be essential for Nrf activity, it was also possible that an independent but essential *nrf* gene as well as *fdhF* had been deleted, or that the parental strain MC4100 was Nrf⁻. To resolve these uncertainties, *cysG*: :Tn5 derivatives of both MC4100 and M9 were constructed by transduction. Glucose- and formate-dependent rates of nitrite reduction of the various MC4100 strains were then compared with strain JCB387 (Table 6). The Nrf activities of both strains M9 *cysG* and MC4100 *cysG* were less than 0.5% of that of JCB387, but cytochrome c_{552} was readily detected.

In a further series of transductions in which JCB387 was used as the donor, a temperature-stabilized, Nrf⁺ Nir⁻ derivative of M9 was isolated. The Nrf activity and concentration of cytochrome c_{552} in these strains were comparable to those of strain JCB387 (Table 6). This result established not only that strain MC4100 and the various *fdhF* mutants derived from it are Nrf⁻, but also that the defective *nrf* genes in these strains are co-transducible with *fdhF*.

Isolation of cosmids which complement some of the Nrf mutations

Attempts to isolate cosmids which transform the Nrf⁻ strain JCB6061 to Nrf⁺ were unsuccessful. Although several transformants were initially Nrf⁺, they were Nrf⁻ when retested. Attempts to subclone complimentary *nrf*⁺ DNA from these clones into multi-copy plasmids were also unsuccessful.

The high frequency of co-transduction of the Nrf⁻ phenotype of FM932 with the *mel*: :Tn10 insertion suggested that the deleted *nrf* genes were probably located between the *fdhF* and *mel* operons and therefore provided an alternative strategy for cloning the deleted genes. The *fdhF* and *mel* genes are 0.8 min or about 40 kb

apart on the *E. coli* chromosome (Fig. 3b): as up to 50 kb of bacterial DNA can be incorporated into a cosmid, it should be possible to clone all of this intervening DNA by isolating cosmids which complement either or both the *mel*: :Tn10 and the *fdhF* defects of strain FM932.

The Fdh⁻ Nrf⁻ Mel⁻ defects of FM932 were transferred by transduction to JCB387 to give JCB3878 which was then transformed with the cosmid library. Approximately 1000 of the several thousand Amp^R clones generated in this experiment were purified onto selective agar plates, screened individually for formate hydrogenlyase activity (*fdhF*⁺) and ability to grow on minimal agar with melibiose as sole carbon source. All of the Mel⁺ or Fhl⁺ clones were then tested for Nrf activity. Nine Mel⁺ clones were all Nrf⁻, but three out of eight Fhl⁺ clones were Nrf⁺. As no Mel⁺ Nrf⁺ clones were isolated, it was apparent the *nrf* genes were closer to *fdhF* than to the *mel* operon.

Cosmid DNA from two independent clones was partially digested with *Sau*3A and fragments in the range 6 to 15 kb were subcloned into the *Bam*HI site of the low copy number vector, pLG339. The ligated DNA was transformed into JCB3878 and, after purification, colonies transformed with Kan^R Tet^S recombinant plasmids were screened for Nrf activity. Five Nrf⁺ clones were isolated from bacteria transformed with DNA from one of the cosmids and one Nrf⁺ clone was derived from the second cosmid. The restriction maps of three of these plasmids, which revealed several common fragments (Fig. 3c), corresponded exactly with the published restriction map of the chromosome close to but on the opposite side of *fdhF* to *mel*. From the size and restriction map of plasmid pLD5, the smallest sub-clone which restored a Nrf⁺ phenotype, it can be concluded that the *nrf* genes deleted from FM932 and JCB3878 are located between coordinates 4365 and 4374 on the Kohara map (Fig. 3b, c; Kohara *et al.*, 1987). None of the sub-clones carried a functional *fdhF*⁺ gene.

Complementation of other mutations with the recombinant plasmids

The only antibiotic resistance marker encoded by pLD5 is Kan^R. The 9 kb *Eco*RI-*Sal*I fragment, which included all of the *E. coli* DNA, was therefore sub-cloned into pBR322 which had been digested with the same enzymes to provide a plasmid which could be used to transform Kan^R Nrf⁻ mutants to Amp^R. The resulting plasmid, pLD5R, was then shown to transform JCB3878 to Nrf⁺.

All of the Nrf⁻ strains with mutations mapped to the 92 to 94 minute region of the chromosome were each transformed with either pLD5 or pLD5R and the Nrf phenotypes of the purified transformants were determined. Strains JCB352, 353, 362 and 602 were all

Table 7. Location by P1-mediated transduction of three groups of *nrf* genes in the 92 to 94 minute region of the *E. coli* chromosome

The numbers of each class of recombinant analysed were typically in the range 72 to 144.

Donor (phenotype) genotype	Recipient (phenotype) genotype	Selected marker	Transduction classes	Frequency (%)
<i>Expt 1</i>				
JCB6069 (Kan ^R Tet ^R) <i>nrf-606 mel::Tn10</i>	M9 Δnir (Nrf ⁺ Fhl ⁻) <i>fdhF nrf</i> ⁺	Tet ^R	Fhl ⁻ Nrf ⁻	10
			Fhl ⁻ Nrf ⁺	39
			Fhl ⁺ Nrf ⁻	51
			Fhl ⁺ Nrf ⁺	0
		Kan ^R	Fhl ⁻ Mel ⁻	38
			Fhl ⁻ Mel ⁺	62
			Fhl ⁺ Mel ⁺	0
			Fhl ⁺ Mel ⁻	0
<i>Expt 2</i>				
FM932 (Fhl ⁻ Nrf ⁻ Tet ^R) <i>fdhF mel::Tn10</i>	JCB606 (Nrf ⁻ Kan ^R) <i>nrf-606</i>	Tet ^R	Fhl ⁻ Kan ^R Nrf ⁻	49
			Fhl ⁻ Kan ^S Nrf ⁻	14
			Fhl ⁺ Kan ^R Nrf ⁻	15
			Fhl ⁺ Kan ^S Nrf ⁺	22
<i>Expt 3</i>				
JCB362 (Nrf ⁻ Amp ^R) <i>nrf-362</i>	FM932 (Fhl ⁻ Nrf ⁻ Tet ^R) <i>fdhF mel::Tn10</i>	Mel ⁺	Amp ^R Fhl ⁺	0
			Amp ^R Fhl ⁻	21
			Amp ^S Fhl ⁺	7
			Amp ^S Fhl ⁻	72
		Amp ^R	Mel ⁺ Fhl ⁺	0
			Mel ⁺ Fhl ⁻	9
			Mel ⁻ Fhl ⁺	82
			Mel ⁻ Fhl ⁻	9
<i>Expt 4</i>				
JCB3513 (Nrf ⁻ Amp ^R) <i>nrf-351</i>	FM932 (Fhl ⁻ Nrf ⁻ Tet ^R) <i>fdhF mel::Tn10</i>	Amp ^R	Fhl ⁺ Mel ⁺	0
			Fhl ⁺ Mel ⁻	63
			Fhl ⁻ Mel ⁺	9
			Fhl ⁻ Mel ⁻	28
		Mel ⁺	Fhl ⁺ Amp ^R	0
			Fhl ⁺ Amp ^S	0
			Fhl ⁻ Amp ^R	8
			Fhl ⁻ Amp ^S	92

transformed to Nrf⁺ by these plasmids, as were the Nrf⁻ HFrH strain CGSC 10235 and MC4100 (Δnir). In contrast, transformants of strains JCB351, 354, 355, 356, 603, 604, 605 and 606 were Nrf⁻. As the mutations carried by the latter strains are all co-transducible with either *mel* or *fdhF*, it is apparent that genes essential for formate-dependent nitrite reduction are located in several separate loci in this region.

At least three loci required for formate-dependent nitrite reduction are located in the 92 to 94 minute region of the E. coli chromosome

Some of the recombinants from the initial transduction experiments provided strains suitable for the fine structure mapping of other *nrf* genes in the 92 to 94 minute region of the chromosome. In the first experiment (Table 7), a *mel::Tn10* derivative of the $\lambda placMu$ insertion in strain JCB606 was used as donor to locate the *nrf-606*

mutation to the opposite side of the *mel* operon to *fdhF*. The recipient, JCB3406, was a Nir⁻ Nrf⁺ derivative of the *fdhF* mutant M9. All of the Kan^R transductants tested were FdhH⁻ (Table 7). In contrast, selection for Tet^R resulted in both Tet^R Kan^R and Tet^R FdhH⁺ transductants, consistent with *mel::Tn10* being the middle marker, but again none of the transductants were both FdhH⁺ and Kan^R, presumably because the distance between either side of these two mutations is too great for all of the intervening DNA to be packaged into bacteriophage P1 (see Fig. 3). In a subsequent experiment, the relative positions of the *nrf-606* and FM932 Nrf⁻ mutations were determined using bacteriophage P1 prepared on the *mel::Tn10* strain, FM932 (experiment 2, Table 7). Of the Tet^R transductants selected, 64% were also Kan^R; the majority of these transductants had also inherited the *fdhF* deletion of the donor. Most significant, however, were the 14% that were Fhl⁻ Kan^S: although these transductants had not inherited the *nrf-606* mu-

tation, they were all nevertheless Nrf⁻. This clearly established that the deletion that resulted in temperature stabilization of the *fdhF* insertion had also resulted in the loss of a gene adjacent to *fdhF* which is essential for Nrf activity. Consistent with this conclusion was the failure to isolate any Fhl⁻ Nrf⁺ transductants in which the *fdhF* deletion had been separated from the defective *nrf* gene.

The transductants isolated from the above experiments were useful for mapping other *nrf* mutations located in the 92 to 94 minute region. In this way, the *nrf*-352, *nrf*-353, *nrf*-362 and *nrf*-379 mutations were located in the same region as the *nrf* deletion in FM932 (see, for example, experiment 3 of Table 7). Finally, a third locus required for Nrf activity which is defective in JCB351 and located between the *fdhF* and *mel* genes was identified (experiment 4 of Table 7; Fig. 3). Further evidence consistent with these proposed map locations came from the isolation of a temperature-stabilized Nrf⁻ FdhH⁻ Mel⁺ deletion derivative of JCB362 and a Nrf⁻ FdhH⁺ Mel⁻ deletion derivative of JCB351.

Discussion

Electron transfer from formate into the Nrf pathway

As energy is conserved during both formate-dependent nitrite and nitrate reduction but not during formate hydrogenlyase activity, it was initially expected that FdhN would be essential for the transfer of electrons from formate to the Nrf complex. Surprisingly, an FdhN⁻ strain was Nrf⁺ and, conversely, the *fdhF* deletion mutant FM932 was Nrf⁻. The simple interpretation of these results, that FdhH is essential for Nrf activity, was known to be incorrect because although transcription of the *fdhF* gene requires the alternative sigma factor, sigma 54 encoded by *rpoN*, an *rpoN* mutant was shown to be Nrf⁺ (Birkman *et al.*, 1987; Page *et al.*, 1990). These uncertainties were resolved by establishing that all three known formate dehydrogenases contribute to the transfer of electrons from formate to nitrite (Table 2) and that the Nrf⁻ defect of strain FM932 is due to the deletion of genes close to but independent of *fdhF* at minute 92.6 on the *E. coli* chromosome (Fig. 3). Further confirmation of these conclusions was provided by ⁷⁵Se labelling experiments, which failed to reveal a fourth, previously undescribed selenoprotein. As FdhO is only poorly expressed (Sawers *et al.*, 1991; Fig. 1) and contributes less than 10% to the total Nrf activity, we conclude that the Nrf pathway primarily exploits the presence of two major formate dehydrogenases involved in other modes of anaerobic growth to transfer electrons from formate to cytochrome *c*₅₅₂.

Rates of formate-dependent nitrite reduction by washed bacteria were decreased by approximately 50%

by mutations resulting in the loss of either of the two major formate dehydrogenases, FdhN or FdhH, and the effects of these mutations were essentially additive (Table 2). If the electron flux from formate to cytochrome *c*₅₅₂ is determined primarily by the sum of the activities of these two enzymes, the consequence of high flux coefficients for these dehydrogenases is that the electron flux to nitrite is relatively insensitive to large changes in the quantity of cytochrome *c*₅₅₂ synthesized. This is particularly significant because, apart from a slight induction of FdhN synthesis by nitrite (Berg & Stewart, 1990), neither of the dehydrogenases is synthesized optimally under growth conditions in which cytochrome *c*₅₅₂ synthesis and Nrf pathway activity are induced. Furthermore, the residual activity of the FdhN⁻ FdhH⁻ double mutant establishes that the poorly expressed FdhO is competent in electron transfer from formate to nitrite as well as to oxygen and nitrate (see Pommier *et al.*, 1992).

Fujita & Sato (1967) reported that nitrite inhibits the production of H₂ from glucose by bacteria grown in the absence of nitrate or nitrite. The production of CO₂ was concomitantly enhanced and nitrite was reduced to ammonia. These early observations can now be interpreted on the basis of competition between hydrogenase 3 and cytochrome *c*₅₅₂ for electrons from FdhH. How electrons are transferred from FdhH to the cytochrome is unclear. Possibly the membrane-spanning proteins HycC and HycD, together with components of the quinone pool, are involved. Alternatively, unknown components of the Nrf pathway might be required to interact directly with FdhH without the involvement of quinones. If so, this could explain why a mutant deficient in ubiquinone and menaquinone synthesis retained 60% of the Nrf activity of the parental strain (Abou-Jaoudé *et al.*, 1979b). The demonstration that the electrogenic FdhN can donate electrons to the Nrf pathway explains how energy is conserved and the involvement of a *b*-type cytochrome in Nrf activity (Motteram *et al.*, 1981; Pope & Cole, 1982; Abou-Jaoudé *et al.*, 1979b). The FdnI component of FdhN is cytochrome *b*₅₅₆ (Berg *et al.*, 1991) and is probably involved in the transfer of electrons from formate to the quinone pool. We suggest that this is the point at which electron transfer to nitrate or nitrite diverges when FdhN is involved in formate oxidation.

Although several of the strains used for the experiments summarized in Table 2 were devoid of both of the two major pathways for nitrite reduction, they were all Nrf⁺, as judged by the test-tube assay for the Nrf phenotypes of recombinants for genetic experiments. This indicates that metabolites of glucose other than formate and NADH can act as electron donors for nitrite reduction. Possible candidates for these alternative electron donors include lactate (Abou-Jaoudé *et al.*,

1979a) and ethanol (Pope & Cole, 1984). Even the *selC* strain, JCB3881, was phenotypically Nrf⁺, as judged by the test-tube assay used to screen recombinants from a genetic experiment in which the concentration of nitrite used was only 0.25 mM. In contrast, *Sel*⁻ strains failed to reduce the 1 mM-nitrite used in initial Nrf tests to screen the libraries of transposon insertion mutants (Table 3).

In summary, we suggest that energy is conserved during nitrite reduction primarily when FdhN donates electrons via the quinone pool to cytochrome *c*₅₅₂. It remains to be established whether the transfer of electrons from FdhH merely serves to detoxify nitrite in the periplasm without the generation of a membrane potential.

Location of genes essential for Nrf activity

Several difficulties had to be overcome for the various *nrf* mutations to be mapped. First, the Nrf phenotype of recombinants could only be scored if both the donor and the recipient were first made Nir⁻ by a series of transduction and screening procedures. Secondly, if either the donor or the recipient was Nir⁺, streptomycin resistance of the recipient could not be used as a counter-selective marker because of the proximity of the streptomycin-resistance determinant, *rpsL*, to the *nir* operon (Macdonald *et al.*, 1985). To overcome this limitation, a *narL::Tn10* mutation was introduced into the recipient strain. A further complication was that many attempts to devise a medium selective for Nrf⁺ recombinants were unsuccessful. The simplest way to distinguish between Nrf⁺ and Nrf⁻ recombinants from genetic crosses was therefore to score for inheritance of antibiotic resistance associated with the insertion mutations. As phenotypic expression is required for full resistance to both ampicillin and kanamycin to develop, it was necessary to arrange for the various *nrf* mutations to be located in the recipient used for conjugation experiments.

The approximate locations of most of the *nrf* mutations have been established in a series of uninterrupted conjugation experiments with an HFr Cavalli derivative as the donor and antibiotic-resistant Nrf⁻ Pur⁻ strains as the recipients. Mutations located in the 92 to 94 minute region were subsequently further sub-divided on the basis of bacteriophage-P1-mediated transduction data. The combined results establish that at least five loci, three of them in the 92 to 94 minute region, are essential for Nrf activity. Experiments currently in progress indicate that the structural gene for cytochrome *c*₅₅₂ is located on plasmid pLD5. Possible functions for the products of the other genes include electron transfer from the various formate dehydrogenases to cytochrome *c*₅₅₂, the attachment of haem to the apoprotein catalysed

by a haem lyase, the transport of a mature cytochrome *c*₅₅₂ across the cytoplasmic membrane or the assembly of cytochrome *c*₅₅₂ from protoporphyrin IX and the apoprotein in the periplasm (Page & Ferguson, 1990; Ramseier *et al.*, 1991).

This research was supported by a Project Grant from the SERC to J.A.C. and SERC Research Studentships to A. Darwin and L. Page. The authors gratefully acknowledge receipt of many useful strains and helpful ideas from B. Bachmann, A. Bock, D. Boxer, S. Busby, M. Chippaux, J. A. DeMoss, M.-A. Mandrand and V. Stewart and thank A. Bock for encouraging P.T. to complete his Diplomarbeit in Birmingham.

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