The Chromosomal Location and Pleiotropic Effects of Mutations of the *nirA*⁺ Gene of *Escherichia coli* K12: The Essential Role of *nirA*⁺ in Nitrite Reduction and in Other Anaerobic Redox Reactions

By B. MARY NEWMAN AND J. A. COLE

Department of Biochemistry, University of Birmingham, P.O. Box 363,
Birmingham B15 2TT

(Received 9 December 1977)

Cytochrome *c*₅₅₂, which has been implicated as an electron carrier for nitrite reduction by *Escherichia coli*, has been separated from NADH-nitrite oxidoreductase activity. The cytochrome is therefore not required for the reduction of nitrite by NADH *in vitro*. Nevertheless, some mutants which were selected by their inability to use nitrite as a nitrogen source during anaerobic growth synthesize neither NADH-nitrite oxidoreductase nor cytochrome *c*₅₅₂. The defects in these mutants are due to mutations in a single gene, *nirA*, which is located at about minute 29 on the recalibrated linkage map. Experiments with an F' plasmid which carries a *nirA*⁺ allele established that *nirA*⁺ is dominant to the defective allele. Other mutants, defective in nitrate reductase activity because of mutations in the *chlA* or *chlB* genes, synthesized nitrite reductase and cytochrome *c*₅₅₂ in the absence of nitrate or nitrite.

A mutant with a defective *fnr* gene was also NirA⁻ and, conversely, *nirA* mutants were Fnr⁻. In a series of transduction experiments, attempts to separate the *nirA* and *fnr* defects were unsuccessful. Furthermore, no complementation was observed when an F' plasmid carrying a defective *nirA* allele was transferred into the *fnr* strain. It is concluded that the *fnr* gene described by Lambden & Guest (1976) is identical to the *nirA* gene and that its product affects the synthesis or assembly of a variety of anaerobic redox enzymes which include nitrite reductase, cytochrome *c*₅₅₂, nitrate reductase, fumarate reductase and formate hydrogenlyase.

INTRODUCTION

Nitrate and nitrite are reduced rapidly by anaerobic cultures of *Escherichia coli* K12, and nitrite can serve as the sole nitrogen source to support growth in a continuous culture (Cole *et al.*, 1974). Presumably, therefore, nitrite is reduced to ammonia. The reduction of nitrate to nitrite is catalysed by a membrane-bound enzyme complex. This reaction is coupled to oxidative phosphorylation and therefore provides energy for anaerobic metabolism (Payne, 1973; Haddock & Kendall-Tobias, 1975; Kroger, 1977). Many species of bacteria reduce nitrite to molecular nitrogen, a process which also generates ATP. Several enzymes which catalyse steps in these reactions are associated with *c*-type cytochromes and with the cytoplasmic membrane (Payne, Riley & Cox, 1971; Matsubara, 1971; Yamanaka & Okuwuki, 1974). In contrast, nitrite reduction by *E. coli* is catalysed by the soluble enzyme NADH-nitrite oxidoreductase (EC 1.6.6.4) and there is as yet no evidence that this reaction is coupled to ATP synthesis. A soluble cytochrome, *c*₅₅₂, has

been implicated in nitrite reduction by this organism because it is synthesized only during anaerobic growth and highest concentrations are found in extracts of bacteria in which nitrite reductase is most active (Fujita, 1966; Fujita & Sato, 1966, 1967; Cole & Wimpenny, 1968). Furthermore, both the activity of the enzyme and the cytochrome concentration were high in extracts of a mutant unable to reduce nitrate because of a defect in the *chlA* gene (O'Hara *et al.*, 1967; Venables, Wimpenny & Cole, 1968; Kavanagh & Cole, 1976; Newman & Cole, 1977). Other mutants with a NirA⁻ phenotype were isolated on the basis of their inability to assimilate nitrite during anaerobic growth: these are deficient in both nitrite reductase activity and cytochrome *c*₅₅₂ (Cole & Ward, 1973). All of these results could be accounted for if cytochrome *c*₅₅₂ were an integral part of the NADH-nitrite oxidoreductase complex, or if the synthesis or activity of the enzyme and the cytochrome were controlled by a common mechanism that is defective in *chlA* and NirA⁻ strains. A third possible explanation is that NirA⁻ strains are defective in more than one gene. To distinguish between these possibilities, we have investigated whether cytochrome *c*₅₅₂ can be separated from nitrite reductase activity without loss of enzyme activity. The biochemical properties of NirA⁺ revertants and *nirA/nirA*⁺ merodiploids have been determined, and the *nirA* gene has been mapped with reference to the *hemA*, *cysB*, *pyrF* and *fnr* genes (Bachmann, Low & Taylor, 1976; Lambden & Guest, 1976).

METHODS

Organisms. The origins of the various strains of *Escherichia coli* K12 are listed in Table 1. Bacteria were stored at 4 °C as stab cultures in nutrient agar deeps (Oxoid CM1).

Media and growth conditions. Nitrogen-free (NF) salts contained (per l distilled water): 10.5 g K₂HPO₄; 4.5 g KH₂PO₄; 1 g trisodium citrate; 125 mg Na₂SO₄; and 5 ml sulphur-free trace metals (Cole *et al.*, 1974). The carbon source was glucose at 27 mM and nitrogen sources were as indicated in the text; these were added aseptically before inoculation from sterile stock solutions of 40 % (w/v) glucose, 20 % (w/v) casein hydrolysate (Oxoid L41), 2 M-NH₄Cl and 1 M-NaNO₂. In some experiments Lennox (1955) broth at the equivalent of single strength replaced the casein hydrolysate. Inocula for batch cultures were aerated at 37 °C for 6 to 8 h in 20 ml Lennox broth, and were transferred to 200 ml of prewarmed NF salts in a 250 ml conical flask. After 16 h at 37 °C without aeration, this culture was transferred to 1.8 l NF salts in a 2 l conical flask.

Escherichia coli HfrC was grown in a continuous culture in minimal medium in which 5 mM-NO₂⁻ was the nitrogen source. At this concentration, nitrite limited growth and the dilution rate was 0.029 h⁻¹ (Cole *et al.*, 1974).

Preparation of cell-free extracts. Bacteria were harvested and washed as described previously (Cole *et al.*, 1974), and resuspended in 2 to 5 vol. TEA or TEM buffer, pH 8.0 (50 mM-Tris/HCl, 5 mM-EDTA and, respectively, 5 mM-ascorbate or 15 mM-mercaptoethanol). Bacteria were broken by passage through a French pressure cell at 4 °C and 69 MPa (10000 lbf in⁻²) or, where indicated, by three 15 s periods of sonication with an MSE sonicator (probe diameter, 1.0 cm) operating at 20000 Hz. High speed supernatant (HSS) and cell membrane (CM) extracts were prepared as described previously (Cole & Rittenberg, 1971).

Enzyme assays. NADH-nitrite oxidoreductase (EC 1.6.6.4) activity was assayed as described by Cole *et al.* (1974) except that open cuvettes at 30 °C were used for some assays. Activities were lower in HSS extracts prepared by sonication and assayed anaerobically at room temperature than in those prepared with the French press and assayed at 30 °C. For this reason, enzyme activities should only be compared within individual experiments. Duplicated assays were reproducible to within 1 % with each HSS extract and to within 20 % with extracts of independent cultures of the same strain.

Formate-nitrate oxidoreductase activity was determined with bacteria which had been grown for 16 h without aeration in 800 ml minimal medium (Newman & Cole, 1977) supplemented with 27 mM-glucose and 6.5 g nutrient broth l⁻¹ (Oxoid CM1) (Lambden & Guest, 1976). Cell membranes resuspended in 50 mM-Na⁺/K⁺ phosphate buffer pH 7.4 were incubated at 37 °C with 40 mM-Na⁺ formate and 40 mM-KNO₃ under a stream of N₂. The protein concentration in the assay tube was in the range 0.5 to 5.0 mg ml⁻¹, depending on the strain used. Samples (50 µl) were transferred at 1 or 2 min intervals for 10 to 20 min into 2.7 ml 1 % (w/v) sulphanilamide in 1 M-HCl. At the end of each set of assays 0.3 ml 0.02 % (w/v) *N*-1-naphthylethylene-diamine dihydrochloride was added. The absorbance at 540 nm, determined after 30 min at room temperature, was proportional to the quantity of nitrite in the sample in the range 0 to 100 nmol. Nitrate reductase activities are expressed as nmol nitrite accumulated min⁻¹ (mg CM protein)⁻¹. The rate of nitrite accumulation was constant during the incubation, and was proportional to the concentration of CM protein

Table 1. *Escherichia coli* strains and their origins

Strain	Phenotype or genotype*	Source or method of isolation
A1004a	<i>ilv met hemA</i>	B. Haddock
c38	<i>chlC</i>	W. A. Venables
CB40	<i>nirA1</i>	See Cole & Ward (1973)
CB64	<i>cysB trp</i>	M. Jones-Mortimer
CB65	<i>cysB trp thyA</i>	Derivative of CB64 spontaneously resistant to 20 µg trimethoprim ml ⁻¹
CB75	<i>pyrF nirA1</i>	Transduce CB242 with P1 propagated on CB247. Select Trp ⁺ ; screen for Pyr ⁻ Nir ⁻
CB98	<i>nirA1 cysB⁺ pro trp his str</i>	See Newman & Cole (1977)
CB201	<i>nirB chl Cys⁻Hfr</i>	<i>N</i> -methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine (NG) mutagenesis of OR56
CB203	NirB ⁻	NG mutagenesis of OR56
CB207	NirA ⁻ Cys ⁻	NG mutagenesis of OR75
CB210	<i>nirA2</i> Cys ⁻	NG mutagenesis of OR75
CB211	<i>nirA3</i> unknown growth requirement	NG mutagenesis of OR75
CB212	NirA ⁻	NG mutagenesis of OR75
CB213	NirB ⁻ Cys ⁻	Ethyl methanesulphonate (EMS) mutagenesis of OR75
CB217	NirB ⁻	EMS mutagenesis of OR75
CB222	<i>nirA1 pro trp recA</i>	See Newman & Cole (1977)
CB222/F'23	<i>nirA1 pro trp recA/F' trp⁺ nirA⁺</i>	Conjugate KLF23/KL181 with CB222. Select Trp ⁺ clone
CB223	<i>nirA1 pro trp recA</i>	Grow CB222/F'23 with 75 µg acridine orange ml ⁻¹ . Plate survivors on to nutrient agar and screen for Trp ⁻ clone
CB242	<i>trp nirA1</i>	Transduce CB64 with P1 propagated on CB40. Select Cys ⁺ ; screen for Nir ⁻
CB247	<i>pyrF</i>	Transduce CB64 with P1 propagated on TKG49. Select Cys ⁺ ; screen for Pyr ⁻
CB248	<i>pyrF trp</i>	Transduce CB64 with P1 propagated on TKG49. Select Cys ⁺ ; screen for Pyr ⁻ Trp ⁻
CB253	<i>trp</i>	Grow CB242 in minimal media with 5 mM-NO ₂ ⁻ and 0.5 mM-NH ₄ ⁺ as nitrogen source. After anaerobic growth, inoculate minimal agar plates containing 5 mM-nitrate as the nitrogen source. Select large colonies after 5 d of anaerobic growth
CB254	<i>fur⁺trp</i>	Transduce JRG861a to Fnr ⁺ with P1 propagated on CB248, selecting for anaerobic growth on lactate/fumarate plates
CB255	<i>fur</i>	Transduce CB248 to Pyr ⁺ with JRG861a-P1. Screen for formate hydrogenlyase-negative clone
Hfr Cavalli	Hfr <i>met</i>	NCIB, Torry Research Station, Aberdeen
JRG861a	<i>gal trpA trpR fnr</i>	J. R. Guest
KLF23/KL181	<i>pyrD thi his trp recA mtl xyl mal gal str/F' trp⁺</i>	B. Bachmann
OR56	Prototrophic Hfr	R. Curtiss III
OR75	Prototrophic Hfr	R. Curtiss III
RK7	Prototroph	MacGregor (1975)
RK7 <i>chlA16</i>	<i>chlA gal</i>	MacGregor (1975)
RK7 <i>chlB36</i>	<i>chlB gal</i>	MacGregor (1975)
RK7 <i>chlC19</i>	<i>chlC gal</i>	MacGregor (1975)
RK7 <i>chlE13</i>	<i>chlE gal</i>	MacGregor (1975)
RK7 <i>chlE44</i>	<i>chlE gal</i>	MacGregor (1975)
TKG49	<i>thr leu ilvA his arg thi pyrF thyA lac qme</i>	J. R. Guest
w1485E	Prototroph, lysogenic for P1kc	J. R. Guest

* The system of genetic nomenclature is based upon the recommendations of Demerec *et al.* (1966).

Table 2. *Separation of nitrite reductase activity from cytochrome c_{552}*

Enzyme activities were determined at 30 °C in open cuvettes, and are expressed as nmol NADH oxidized min⁻¹ (mg protein)⁻¹.

Sample	Total protein (mg)	Cytochrome c_{552}		Nitrite reductase	
		nmol	Recovery (%)	Activity	Recovery (%)
HSS extract	1120	1010	100	42	100
(NH ₄) ₂ SO ₄ supernatant	714	775	77	0	0
Desalted (NH ₄) ₂ SO ₄ pellet	429	188	19	53	45
DE52 pool	45	0	0	169	16

in the range 0 to 1.0 mg ml⁻¹ for wild-type strains and in the range 1 to 5 mg ml⁻¹ for Fnr⁻, NirA⁻ and chlorate-resistant mutants. The specific activities of independently prepared samples of membranes from wild-type strains were reproducible to within 20 %, but far greater variations were found for mutant strains: this was probably caused by inactivation of the nitrate reductase complex during the preparative procedures (see also Lambden & Guest, 1976). Nevertheless, the differences between the activities of wild-type and mutant strains were sufficiently great for these assays to yield reliable data.

Formate hydrogenlyase activity was determined qualitatively by growing bacteria in test-tubes containing 5 ml Lennox broth, 27 mM-glucose and an inverted Durham tube. A clearly visible bubble of H₂ had accumulated in wild-type cultures after 18 to 24 h at 37 °C. No gas bubble was visible for at least 48 h when the *fur* mutant JRG861a was tested.

Strains were tested for their ability to reduce nitrite as described by Cole & Ward (1973), but with the following modifications. Bacteria from single colonies were inoculated into 1 ml Lennox broth. After 16 h at 37 °C, 4 ml NF salts supplemented with 27 mM-glucose, 14 mM-NH₄⁺, 5 mM-NaNO₂ and required amino acids at 20 µg ml⁻¹ was added. After 3 h at 37 °C and at subsequent hourly intervals, drops from each culture were tested for the presence of nitrite. Wild-type bacteria had usually reduced the nitrite after 3 to 5 h, but nitrite remained in Nir⁻ cultures after 6 h.

Cytochrome c_{552} was assayed as described previously (Cole *et al.*, 1974). Difference spectra of cytochromes at -196 °C were obtained by immersing cuvettes containing the oxidized and reduced samples in liquid nitrogen and recording the spectrum with a Perkin-Elmer 356 double-beam spectrophotometer equipped with a cryostat attachment.

Separation of nitrite reductase from cytochrome c_{552} . Proteins precipitated from an HSS extract of *E. coli* HfrC by 40 % saturated (NH₄)₂SO₄ in TEA were collected by centrifugation for 10 min at 10000 *g* and redissolved in TEM pH 8.0. After passage through a column of Sephadex G-25 (25.0 × 4.0 cm diam.), the desalted proteins were applied to a column of Whatman DE52 cellulose (17.5 × 2.8 cm diam.) equilibrated with TEM pH 8.0. Nitrite reductase was most active in fractions 72 to 80 (see Fig. 1): these fractions were therefore combined to give the DE52 pool.

Genetic methods. Lennox (1955) broth was used for conjugation and transduction experiments, as described by Newman & Cole (1977). Unless otherwise stated, a virulent derivative of bacteriophage P1kc was used to prepare lysates for transduction experiments.

Mutants unable to reduce nitrite were isolated as described by Cole & Ward (1973).

To isolate homogenotized *nirA* merodiploid strains, CB98 *pro his trp nirA/F'nirA⁺trp⁺* was grown aerobically for 16 h in minimal medium supplemented with proline and histidine and then 10⁻³ to 10⁻⁵ dilutions were plated on to a similar medium containing 1.25 % (w/v) agar to obtain single colonies. Twenty-five Nir⁻ isolates were detected amongst 700 colonies that were tested, and five of these were retained for complementation experiments.

RESULTS

Separation of cytochrome c_{552} from nitrite reductase activity

Escherichia coli strain HfrC was grown in anaerobic continuous culture with nitrite as the nitrogen source. Bacteria suspended in TEA were broken with the French pressure cell and HSS extracts were prepared. Nitrite reductase activity was completely precipitated by 40 % saturated (NH₄)₂SO₄, but 80 % of the cytochrome c_{552} remained soluble (Table 2). The (NH₄)₂SO₄ pellet was resuspended in TEM and desalted by passage through a Sephadex G-25 column equilibrated with TEM. Active fractions were then applied to an anion

Fig. 1. Separation of nitrite reductase activity (○) from cytochrome c_{552} (●) by Whatman DE52 anion exchange chromatography. For experimental details, see Methods.

Table 3. Nitrite reductase activity and cytochrome c_{552} concentration of wild-type and mutant strains of *E. coli*

Escherichia coli strains were grown without aeration in NF salts supplemented with Lennox (1955) broth, 20 mM- NH_4^+ and 2 mM- NO_2^- . HSS extracts were prepared after cells had been broken by sonication. Nitrite reductase activities were assayed in anaerobic cuvettes at room temperature. Activities are expressed as nmol NADH oxidized min^{-1} (mg protein) $^{-1}$, and cytochrome c_{552} concentrations as pmol (mg protein) $^{-1}$. All results represent the mean of at least two experiments.

Strain	Nitrite reductase	Cytochrome c_{552}	Phenotype
CB201	2	35	NirB ⁻
CB203	5	55	NirB ⁻
CB207	5	Broad peak from 550 to 560 nm	NirA ⁻
CB210	6	0	NirA ⁻
CB211	8	0	NirA ⁻
CB212	30	0	NirA ⁻
CB213	0	40	NirB ⁻
CB217	0	13	NirB ⁻
OR75	200	33	Nir ⁺
OR56	90	35	Nir ⁺

exchange column and eluted with a linear gradient of KCl in TEM. The cytochrome was eluted as a well-defined peak by 0.16 M-KCl, before the peak of enzyme activity (Fig. 1). The fractions containing most of the enzyme activity and the highest concentrations of cytochrome c_{552} were pooled and assayed alone and together for both proteins. Partially purified nitrite reductase was uncontaminated with cytochrome c_{552} and its activity was neither inhibited nor enhanced by the cytochrome fraction. The specific activity of nitrite reductase had increased fourfold during the separation procedures, and 16 % of the original activity was recovered. It is concluded that cytochrome c_{552} is not a component of the NADH-nitrite oxidoreductase complex.

Properties of mutants unable to reduce nitrite

Eight newly isolated mutants defective in their ability to reduce nitrite were grown anaerobically with nitrite. The activity of nitrite reductase and the concentration of cytochrome c_{552} in HSS extracts of these strains are shown in Table 3. Nitrite reductase activity

was low or absent in seven of these mutants, but extracts of CB212 retained some activity which was less than 50 % of the lowest activity detected in wild-type bacteria. Four of the mutants, CB207, CB210, CB211 and CB212, were similar to the original NirA⁻ isolates in that cytochrome *c*₅₅₂ could not be detected. These strains are therefore designated NirA⁻. Cytochrome *c*₅₅₂ was detected in strains CB201, CB203, CB213 and CB217. These isolates represent a new class of mutant and are designated NirB⁻. Their biochemical and genetic properties will be reported elsewhere.

Chromosomal location of the nirA gene

Bacteriophage P1 was propagated on strain CB40 *cysB*⁺*nirA*1 and the lysate was used to transduce strain CB64 to Cys⁺. In one experiment, 196 Cys⁺ transductants were tested for their ability to reduce nitrite: 4 were Nir⁻ and 192 were Nir⁺. This low cotransduction frequency (2 %) suggested that the *nirA*1 defect is located about 1.5 min away from *cysB*. The frequency of cotransduction of *nirA* with *hemA* and *pyrF* genes located on either side of *cysB* was therefore investigated. The *hemA* mutant A1004a was used as the recipient in a transduction with strain CB98 *nirA*1 as the donor, and 200 Hem⁺ transductants were tested for their ability to reduce nitrite. None were Nir⁻. In duplicate experiments with the *cysB*⁺*trp*⁺*pyrF* strain CB247 as the recipient and CB242 *nirA*1 *trp* as the donor, 17 out of 297 and 5 out of 80 Pyr⁺ recombinants were Nir⁻. The cotransduction frequency between *pyrF*⁺ and *nirA*1 is therefore about 6 %. If it is assumed that the Wu (1966) mapping function is applicable to these low cotransduction frequencies, [it can be calculated that the *nirA* gene is located approximately 1.2 min away from the *pyrF* gene. Only 1 of the 22 Pyr⁺Nir⁻ transductants had inherited the donor Trp⁻ phenotype. The *nirA* gene is therefore on the opposite side of *pyrF* to the *cysB* gene and the tryptophan operon and is located at approximately minute 29.2 on the linkage map of Bachmann *et al.* (1976).

Two other NirA⁻ mutants, CB210 and CB211, were used as donors to transduce CB247 to Pyr⁺. In each experiment at least 2 out of 100 PyrF⁺ recombinants were Nir⁻. Furthermore, the Nir⁻ defect in CB210 did not cotransduce with *hemA*⁺. The mutations in CB210 and CB211 are therefore located in the same 2 min segment of the chromosome as the *nirA*1 mutation in strains CB40, CB98 and CB242.

Pleiotropic effects of a nirA mutation

Four *cysB*⁺*nirA*1 recombinants were purified from the transduction with CB40 as the donor and CB64 as the recipient. Cell-free extracts of these strains prepared after anaerobic growth with nitrite were deficient in both nitrite reductase activity and cytochrome *c*₅₅₂ (Table 4). Similar data were obtained with Pyr⁺Nir⁻ recombinants from transductions with strains CB210, CB211 and CB242 as the donor and CB247 as the recipient. In contrast, Pyr⁺Nir⁺ transductants synthesized both proteins (Table 4). It is apparent, therefore, that the *nirA* gene affects the synthesis of both nitrite reductase and cytochrome *c*₅₅₂.

To exclude the possibility that all three NirA⁻ strains carry mutations in two or more closely linked genes or that all of the strains have deletions covering more than one gene, a spontaneous Nir⁺ revertant of CB242 *nirA* was isolated. The nitrite reductase activity of this revertant, CB253, was similar to that of the original wild-type. Furthermore, the low temperature cytochrome spectrum of an HSS extract of CB253 was identical to that of the wild-type with an absorbance maximum at 550 nm as well as at 556 and 559 nm (Fig. 2). In contrast, no absorbance maximum at 550 nm was observed in spectra of *nirA* strains (Fig. 2). Thus cytochrome *c*₅₅₂ synthesis was restored to a revertant that had been selected on the basis of its ability to reduce nitrite.

PyrF⁺ transductants were selected from two experiments with the revertant CB253 as the donor and either a *pyrF nirA*1 or a *pyrF nirA*⁺ strain as the recipient. When strain CB247 *pyrF nirA*⁺ was the recipient, 100 out of 100 Pyr⁺ transductants were Nir⁺. The revertant

Table 4. Nitrite reductase activity and cytochrome c_{552} concentration in *nirA*⁺ and *nirA* transductants

Escherichia coli strains were grown without aeration in minimal medium containing 1 g casein hydrolysate l⁻¹, 20 mM-NH₄⁺ and 2.5 mM-NO₃⁻. The activity of nitrite reductase was assayed at 30 °C with open cuvettes. Nitrite reductase activities are expressed as nmol NADH oxidized min⁻¹ (mg protein)⁻¹, and cytochrome c_{552} concentrations as pmol (mg protein)⁻¹. Strains CB64, CB75, CB242, CB247, CB248 and CB253 share a common genetic background.

Donor	Recipient	Recombinant phenotype	Nitrite reductase	Cytochrome <i>c</i> ₅₅₂
CB40 Nir ⁻	CB64 Nir ⁺	Cys ⁺ Nir ⁻ (1)	34	< 10
		(2)	28	< 10
		(3)	31	< 10
		(4)	22	< 10
CB242 <i>nirA1</i>	CB247 <i>nir</i> ⁺	Pyr ⁺ Nir ⁻ (1)	0	< 10
		(2)	9	< 10
CB211 <i>nirA3</i>	CB247 <i>nir</i> ⁺	Pyr ⁺ Nir ⁻ (1)	0	< 10
		(2)	34	< 10
		Pyr ⁺ Nir ⁺	555	206
CB210 <i>nirA2</i>	CB247 <i>nir</i> ⁺	Pyr ⁺ Nir ⁻ (1)	0	< 10
		(2)	0	< 10
		Pyr ⁺ Nir ⁺	292	172
CB253 <i>nir</i> ⁺	CB75 <i>nirA1</i>	Pyr ⁺ Nir ⁺ (1)	716	82
		(2)	993	183
		(3)	799	92
JRG861a <i>fnr</i>	CB248 <i>nir</i> ⁺	Pyr ⁺ Fnr ⁻ (1)	0	< 10
		(2)	0	< 10
		(3)	3	< 10
Control: JRG861a		—	5	< 10
Control: CB248		—	125	124

Fig. 2. Difference spectra of reduced minus oxidized HSS extracts of the *nirA1* strain CB242 and the revertant CB253. Samples were frozen in liquid nitrogen and spectra were recorded with a Perkin-Elmer 356 double-beam spectrophotometer. The vertical bar corresponds to an absorbance increment of 0.1 for the revertant sample, and 0.003 for the mutant sample (protein concentrations were 12.5 and 4.3 mg ml⁻¹, respectively).

Table 5. *Nitrite reductase activity and cytochrome c_{552} concentration in chlorate-resistant mutants*

Bacteria were grown in NF salts with 1 g casein hydrolysate l^{-1} and, where indicated, 10 mM- KNO_3 or 5 mM- $NaNO_2$. Cell-free extracts were prepared with the French pressure cell, and nitrite reductase activities were determined at 30 °C with open cuvettes. Each line of data is derived from a single independent culture. Nitrite reductase activities are expressed as nmol NADH oxidized min^{-1} (mg protein) $^{-1}$, and cytochrome c_{552} concentrations as pmol (mg protein) $^{-1}$.

Strain	Additional N source	Nitrite reductase	Cytochrome c_{552}
RK7 (wild-type)	None	62	< 10
		54	< 10
RK7 <i>chlA16</i>	None	536	103
		478	97
RK7 <i>chlB36</i>	None	625	118
		438	49
RK7 <i>chlC19</i>	None	71	< 10
		75	< 10
RK7 <i>chlE13</i>	None	45	< 10
		15	< 10
RK7 <i>chlE44</i>	None	52	< 10
		43	< 10
RK7 <i>chlA16</i>	Nitrate	589	< 10
RK7 <i>chlB36</i>	Nitrate	500	< 10
RK7 <i>chlC19</i>	Nitrate	293	35
RK7 <i>chlE13</i>	Nitrate	383	< 10
RK7 <i>chlE44</i>	Nitrate	359	37
RK7 <i>chlA16</i>	Nitrite	446	< 10
RK7 <i>chlB36</i>	Nitrite	361	< 10
c38 <i>chlC</i>	None	151	< 10
		113	60
c38 <i>chlC</i>	Nitrate	348	< 10

CB253 therefore no longer carries a *nirA* defect that can readily be segregated from the reversion site. Conversely, when CB75 *pyrF nirA1* was the recipient, 94 out of 100 Pyr^+ transductants were still Nir^- : none of the six that were Nir^+ had inherited the Trp^- phenotype of the donor. Three of these Pyr^+Nir^+ transductants were assayed for nitrite reductase activity and cytochrome c_{552} synthesis: all were similar to the wild-type in both respects (Table 4). The cotransduction frequency of the Nir^+ reversion site with *pyrF* $^+$ is therefore 6 %, and the mutation must lie on the opposite side of *pyrF* with respect to the tryptophan operon. The reversion site is therefore in, or extremely close to the *nirA* gene.

Dominance of the nirA⁺ allele

The preceding experiments established that defects in the *nirA* gene result in the loss of at least two proteins. It is possible, therefore, that *nirA* $^+$ is a regulatory gene coding for a positive control protein or a repressor. If the second alternative is correct, it would be necessary to suggest that *nirA* mutants synthesize a superrepressor similar to that specified by the *lac* i^s allele (Jacob & Monod, 1961). Dominance tests were used to distinguish between these two possibilities.

The *nirA* ^+trp $^+$ plasmid F'23 was introduced into CB222, a *recA* derivative of CB98 *nirA trp*, and the nitrite reductase activity and cytochrome c_{552} concentration in the merodiploid, the original mutant and a cured derivative of the merodiploid were determined. Only the merodiploid strain reduced nitrite during growth, and nitrite reductase activity and cytochrome c_{552} were detected only in HSS extracts of this strain.

At the end of growth, 25 % of the single colony isolates from the CB222/F'23 culture were Trp^+ and were able to donate this phenotype to CB64 *trp* by conjugation. Thus a minority

Table 6. Nitrite reduction and formate hydrogenlyase activity during growth, and formate-nitrate oxidoreductase activity of various *nirA*, *fnr* and wild-type strains

Formate hydrogenlyase activities were recorded after 30 h at 37 °C; each culture was then supplemented with 27 mM-glucose and incubated for a further 50 h at 37 °C. Data for formate-nitrate oxidoreductase activities [expressed as nmol NO₂⁻ formed min⁻¹ (mg protein)⁻¹] are for single experiments: activities of 2.0 and 22.5 nmol NO₂⁻ formed min⁻¹ (mg protein)⁻¹ were found for independent cultures of strains JRG861a and CB248, respectively.

Strain	Parent	Relevant genotype	Nitrite re-duction during growth	Gas production by formate hydrogenlyase		Formate-nitrate oxido-reductase
				After 30 h	After 80 h	
JRG861a	JRG861a	<i>fnr</i>	—	—	+	10.8
CB254	JRG861a	<i>fnr</i> ⁺	+	+	+++	125.0
CB64	CB64	<i>nirA</i> ⁺ <i>fnr</i> ⁺	+	+	++	ND*
CB242	CB64	<i>nirA1</i>	—	—	—	3.7
CB242/F'23	CB64	<i>nirA1</i> /F' <i>nirA</i> ⁺	+	+	+++	30.0
CB248	CB64	<i>nirA</i> ⁺	+	+	+++	21.3
CB253	CB64	revertant of <i>nirA1</i>	+	+	+++	18.3
CB255	CB64	<i>fnr</i>	—	—	—	2.2
CB210	OR75	<i>nirA2</i>	—	—	—	ND
CB211	OR75	<i>nirA3</i>	—	—	—	ND

* ND, Not determined.

of the bacteria had retained the plasmid. For this reason, the nitrite reductase activity of this culture was lower than that of a haploid *nirA*⁺ strain. The *nirA*⁺ plasmid F'23 therefore restored nitrite reductase activity and cytochrome *c*₅₅₂ synthesis to a *nirA* strain, and the *nirA*⁺ allele is dominant to *nirA1*.

Nitrite reductase and cytochrome *c*₅₅₂ in chlorate-resistant mutants

Previous reports that nitrite reductase and cytochrome *c*₅₅₂ are derepressed in a *chlA* mutant suggest that there is a common component which regulates the synthesis, assembly or activity of both of these proteins as well as the nitrate reductase complex (O'Hara *et al.*, 1967; Venables *et al.*, 1968). The effects of mutations in other *chl* genes were therefore investigated.

Nitrite reductase and cytochrome *c*₅₅₂ were apparently derepressed during growth of *chlA* and *chlB* mutants without nitrate or nitrite, but both oxidizing agents repressed the synthesis of cytochrome *c*₅₅₂ (Table 5). The regulation of the synthesis of both proteins in *chlC* and *chlE* strains was similar to that of the wild-type. No nitrite accumulated in the growth medium when strains *chlC38*, *chlE13* and *chlE44* were grown with 10 mM-KNO₃. The derepression of nitrite reductase in these strains is therefore probably caused by nitrate rather than its reduction product, nitrite.

Relative positions of the *nirA* and *fnr* genes

At least three genes, *nirA*, *chlA* and *chlB*, affect the synthesis of both nitrite reductase and cytochrome *c*₅₅₂. Lambden & Guest (1976) have described other mutants with defects in the *fnr* gene which are also defective in the synthesis or activity of several proteins that catalyse anaerobic oxidation-reduction reactions. Furthermore, the *fnr* gene, like the *nirA* gene, is 5 to 9 % cotransducible with *pyrF*⁺ and is located at approximately minute 29 on the recalibrated *E. coli* linkage map (Lambden & Guest, 1976). It was therefore of interest to determine the relative positions of the *nirA* and *fnr* genes to investigate whether they

might be sufficiently close to be part of an operon concerned with anaerobic metabolism or its regulation.

Preliminary experiments established that the *fnr* strain JRG861a was unable to reduce nitrite and, conversely, that the *nirA* strain CB242 was Fnr⁻ (Table 6). Furthermore, a variety of other *nirA* strains were Fnr⁻, but the Nir⁺ revertant CB253 and the *nirA/nirA*⁺ merodiploid CB242/F'23 were Fnr⁺ (Table 6). Strain CB248 was transduced with phage P1 which had been propagated on JRG861a, and 200 Pyr⁺ transductants from each of two independent experiments were scored for their Nir and Fnr phenotypes. In the first experiment, 197 Pyr⁺ transductants were both Fnr⁺ and Nir⁺, and 3 were both Fnr⁻ and Nir⁻ (Table 4). In the second experiment, 192 Pyr⁺ transductants were also Fnr⁺Nir⁺, and 8 were Fnr⁻Nir⁻. For the reciprocal cross, phage propagated on strain CB248 was used to transduce JRG861a to Fnr⁺, and transductants were selected by their ability to grow anaerobically with lactate plus fumarate as the source of carbon and energy. Two hundred Fnr⁺ transductants were all Nir⁺. It was possible, therefore, that the NirA⁻ and Fnr⁻ phenotypes result from defects in a single gene which has previously been designated either *nirA* or *fnr*.

Lack of complementation between nirA and fnr strains

Two approaches were used in attempts to demonstrate complementation between *nirA* and *fnr* mutant strains. If the NirA⁻ and Fnr⁻ phenotypes result from defects in separate genes, one would predict that abortive transductants would be generated when an *fnr* strain was transduced with phage P1 that had been propagated on a *nirA* strain. Similarly, wild-type transconjugants should be generated following the transfer of an F' plasmid carrying a defective *nirA* gene into an *fnr* strain.

A lysate of avirulent bacteriophage P1kc, generated by growing strain w1485E aerobically for 16 h in Lennox broth, was propagated on strain CB242 *nirA gal*⁺ and used at a multiplicity of infection of 2 to transduce JRG861a *fnr gal* to Fnr⁺ or Gal⁺. Fnr⁺ transductants were selected on glycerol/fumarate plates (Lambden & Guest, 1976). Colonies of three different sizes had formed after 4 d of anaerobic growth. The frequency of occurrence of the largest colony type was 3.9 to 8.0 per 10⁸ phage particles compared with a frequency of 40 to 190 Gal⁺ transductants per 10⁸ phage from the same experiments. Although far more of the small and very small colonies were seen, similar numbers formed on control plates spread with JRG861a which had not been infected with P1kc. The large colonies from three such experiments (60 isolates) were purified and tested for their ability to reduce nitrite and to catalyse the formate hydrogenlyase reaction. All were Nir⁺Fnr⁺. Similarly, small and very small colonies from both transduced and control samples of JRG861a were all Nir⁻Fnr⁻. Although it is impossible to state that abortive transductants were not seen in these experiments, the very close proximity of the Nir⁻ and Fnr⁻ defects carried by the donor and recipient strains has been confirmed.

The merodiploid CB98 *nirA trp*/F'*nirA*⁺*trp*⁺ and five homogenotized Nir⁻ derivatives were incubated for 30 min in Lennox broth with strain JRG861a, and Trp⁺ transconjugants were selected. The Nir and Fnr phenotypes of 100 purified transconjugants were determined after their fertility had been established by transferring the *cysB*⁺*trp*⁺ alleles into strain CB65 *cysB trp thyA*. Thirty transconjugants which had acquired the F'*nirA*⁺*trp*⁺ plasmid were wild-type for formate hydrogenlyase and nitrite reductase activities and formed only large colonies during anaerobic growth on glycerol/fumarate plates. In contrast, 70 transconjugants which had inherited the F'*nirA* *trp*⁺ plasmids were Nir⁻: formate hydrogenlyase was either less active or absent and only a few large colonies formed on glycerol/fumarate plates. These large colonies were subsequently shown to be Nir⁺Fnr⁺ recombinant derivatives of the original Nir⁻Fnr⁻ transconjugants: their occurrence is positive evidence that the F' plasmids carried a defective *nirA* gene rather than a small internal deletion.

DISCUSSION

Although it has previously been suggested that cytochrome c_{552} is involved in nitrite reduction by *E. coli*, the separation of these two proteins without a concomitant decrease in the specific activity of the enzyme has now established that the cytochrome is not required for the reduction of nitrite by NADH *in vitro* (see Cole & Wimpenny, 1968). However, *chlA* and *chlB* mutants are derepressed for both proteins in the absence of nitrate or nitrite, and *nirA* mutants which carry a defect in a single gene lack both the enzyme and the cytochrome. Common components therefore regulate the synthesis or assembly of both proteins. The *nirA* gene is located at approximately minute 29, distal to *cysB* rather than adjacent to *hemA* as shown on the current linkage map (Bachmann *et al.*, 1976). The low frequency of recombination and lack of complementation between a NirA⁻ strain and an Fnr⁻ strain indicate that the *nirA* and *fnr* genes are probably identical.

Several possible explanations for the pleiotropic phenotypes of *nirA* mutants have been considered. One is that all *nirA* strains carry highly polar mutations in an operator proximal gene of an operon which codes for nitrite reductase and cytochrome c_{552} . This is unlikely, however, because the rates of synthesis of these two proteins are not always coordinately regulated as would be expected if they were under the control of a common promoter, operator or attenuator (see Table 5 and Cole *et al.*, 1974). Furthermore, preliminary data not presented here indicate that the probable structural gene for nitrite reductase, *nirB*, is unlinked to *nirA*.

Any model postulated to explain the regulation of nitrite reductase and cytochrome c_{552} synthesis must be consistent with the observations that these proteins are induced during anaerobic growth but repressed when cultures are aerated; that they are induced by low concentrations of nitrate or nitrite, but not by high concentrations of these oxidants; and that neither nitrate nor nitrite are required for them to be synthesized by *chlA* and *chlB* mutants (Cole & Wimpenny, 1968; Cole *et al.*, 1974; Table 5). Furthermore, the model should be compatible with the defects in fumarate reductase, nitrate reductase (EC 1.6.6.1), hydrogenase (EC 1.12.7.1) and formate hydrogenlyase that were described by Lambden & Guest (1976). One possibility is that the *nirA*⁺ gene codes for a positive regulator protein that is essential for the synthesis of a variety of enzymes involved in anaerobic redox reactions. It is further suggested that the activation of genes for nitrite reductase and cytochrome c_{552} by the *nirA*⁺ product is prevented by another factor, B, which is involved in nitrate reduction and is missing or defective in *chlA* and *chlB* strains. It is pertinent that Pateman & Cove (1967) have postulated that the synthesis of nitrite reductase in *Aspergillus nidulans* is also regulated by a component of nitrate reductase.

Alternative explanations of the data cannot be excluded because the dominance of a *nirA*⁺ to a *nirA* allele is equally consistent with a post-translational function for the *nirA*⁺ gene product (protein A). For example, although the multiple defects that arise from an *fnr* or a *nirA* mutation exclude the possibility that *nirA* is a structural gene for the affected enzymes, they are consistent with the suggestion that A is an enzyme which is involved in the synthesis of a prosthetic group – possibly a haem group – common to each of these enzymes. Until the postulated proteins A and B have been isolated, it will be impossible to determine how effector molecules such as oxygen, nitrate and nitrite regulate anaerobic oxidation–reduction processes.

We are grateful to Professors P. H. Clarke and H. L. Kornberg for suggesting possible cytoplasmic roles for the *nirA* gene product; to Dr J. R. Guest for extensive discussions and for providing the *fnr* mutant strain, and to Miss P. Branson for excellent technical assistance. B.M.N. was supported by a Research Studentship from the Science Research Council.

REFERENCES

- BACHMANN, B. J., LOW, K. B. & TAYLOR, A. L. (1976). Recalibrated linkage map of *Escherichia coli* K12. *Bacteriological Reviews* **40**, 116-167.
- COLE, J. A. & RITTENBERG, S. C. (1971). A comparison of respiratory processes in *Spirillum volutans*, *Spirillum itersonii* and *Spirillum serpens*. *Journal of General Microbiology* **69**, 373-383.
- COLE, J. A. & WARD, F. B. (1973). Nitrite reductase-deficient mutants of *Escherichia coli* K12. *Journal of General Microbiology* **76**, 21-29.
- COLE, J. A. & WIMPENNY, J. W. T. (1968). Metabolic pathways for nitrate reduction in *Escherichia coli*. *Biochimica et biophysica acta* **162**, 39-48.
- COLE, J. A., COLEMAN, K. J., COMPTON, B. E., KAVANAGH, B. M. & KEEVIL, C. W. (1974). Nitrite and ammonia assimilation by anaerobic continuous cultures of *Escherichia coli*. *Journal of General Microbiology* **85**, 11-22.
- DEMEREK, M., ADELBERG, E. A., CLARK, A. J. & HARTMAN, P. E. (1966). A proposal for a uniform nomenclature in bacterial genetics. *Genetics* **54**, 61-76.
- FUJITA, T. (1966). Studies on soluble cytochromes in Enterobacteriaceae. I. Detection, purification and properties of cytochrome c_{552} in anaerobically grown cells. *Journal of Biochemistry* **60**, 204-215.
- FUJITA, T. & SATO, R. (1966). Studies on soluble cytochromes in Enterobacteriaceae. IV. Possible involvement of cytochrome c_{552} in anaerobic nitrite metabolism. *Journal of Biochemistry* **60**, 691-700.
- FUJITA, T. & SATO, R. (1967). Studies on soluble cytochromes in Enterobacteriaceae. V. Nitrite-dependent gas evolution in cells containing cytochrome c_{552} . *Journal of Biochemistry* **62**, 230-238.
- HADDOCK, B. A. & KENDALL-TOBIAS, M. W. (1975). Functional anaerobic electron transport linked to the reduction of nitrate and fumarate in membranes from *Escherichia coli* as demonstrated by quenching of atebirin fluorescence. *Biochemical Journal* **152**, 655-659.
- JACOB, F. & MONOD, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *Journal of Molecular Biology* **3**, 318-356.
- KAVANAGH, B. M. & COLE, J. A. (1976). The regulation of nitrogen metabolism in batch and continuous cultures of *Escherichia coli*: facts and artefacts. In *Continuous Culture: Applications and New Fields*, Proceedings of the 6th International Symposium on Continuous Culture of Microorganisms, pp. 184-194. Edited by A. C. R. Dean, D. C. Ellwood, C. G. J. Evans and J. Melling. Chichester: Ellis Horwood.
- KROGER, A. (1977). Phosphorylative electron transport with fumarate and nitrate as terminal hydrogen acceptors. *Symposia of the Society for General Microbiology* **27**, 61-93.
- LAMB DEN, P. R. & GUEST, J. R. (1976). Mutants of *Escherichia coli* K12 unable to use fumarate as an anaerobic electron acceptor. *Journal of General Microbiology* **97**, 145-160.
- LENNOX, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190-206.
- MACGREGOR, C. H. (1975). Synthesis of nitrate reductase components in chlorate-resistant mutants of *Escherichia coli*. *Journal of Bacteriology* **121**, 1117-1121.
- MATSUBARA, T. (1971). Studies on denitrification. XIII. Some properties of the N_2O -anaerobically grown cell. *Journal of Biochemistry* **69**, 991-1001.
- NEWMAN, B. M. & COLE, J. A. (1977). Lack of a regulatory function for glutamine synthetase protein in the synthesis of glutamate dehydrogenase and nitrite reductase in *Escherichia coli* K12. *Journal of General Microbiology* **98**, 369-377.
- O'HARA, J., GRAY, C., PUIG, J. & PICHINOTY, F. (1967). Defects in formate hydrogenlyase in nitrate-negative mutants of *Escherichia coli*. *Biochemical and Biophysical Research Communications* **28**, 951-957.
- PATEMAN, J. A. & COVE, D. J. (1967). Regulation of nitrate reduction in *Aspergillus nidulans*. *Nature, London* **215**, 1234-1237.
- PAYNE, W. J. (1973). Reduction of nitrogenous oxides by microorganisms. *Bacteriological Reviews* **40**, 409-452.
- PAYNE, W. J., RILEY, P. S. & COX, C. D. (1971). Separate nitrite, nitric oxide and nitrous oxide reducing fractions from *Pseudomonas perfectomarinus*. *Journal of Bacteriology* **106**, 356-361.
- VENABLES, W. A., WIMPENNY, J. W. T. & COLE, J. A. (1968). Enzymic properties of a mutant of *Escherichia coli* K12 lacking nitrate reductase. *Archiv für Mikrobiologie* **63**, 117-121.
- WU, T. T. (1966). A model for three-point analysis of random general transduction. *Genetics* **54**, 405-410.
- YAMANAKA, T. & OKUWUKI, K. (1974). Cytochromes. In *Microbial Iron Metabolism, A Comprehensive Treatise*, pp. 349-400. Edited by J. B. Neilands. New York: Academic Press.