Isolation, Characterization and Complementation Analysis of *nirB* Mutants of *Escherichia coli* Deficient Only in NADH-dependent Nitrite Reductase Activity

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Mutants have been isolated which lack NADH-dependent nitrite reductase activity but retain NADPH-dependent sulphite reductase and formate hydrogenlyase activities. These NirB[−] strains synthesize cytochrome *c*₅₅₂ and grow normally on anaerobic glycerol–fumarate plates. The defects map in a gene, *nirB*, which is extremely close to *cysG*, the gene order being *crp*, *nirB*, *cysG*, *aroB*. Complementation studies established that *nirB*⁺ and *cysG*⁺ can be expressed independently. The data strongly suggest that *nirB* is the structural gene for the 88 kDal NADH-dependent nitrite oxidoreductase apoprotein (EC 1.6.6.4).

The *nirB* gene is apparently defective in the previously described *nirD* mutant, LCB82. The *nirH* mutant, LCB197, was unable to use formate as electron donor for nitrite reduction, but NADH-dependent nitrite reductase was extremely active in this strain and a normal content of cytochrome *c*₅₅₂ was detected. Strains carrying a *nirE*, *nirF* or *nirG* mutation gave normal rates of nitrite reduction by glucose, formate or NADH.

INTRODUCTION

Anaerobic cultures of *Escherichia coli* K12 reduce nitrite rapidly to ammonia (Cole, 1978). The most active of the three enzymes involved in this reaction is an NADH-dependent nitrite reductase (EC 1.6.6.4) which, in a typical wild-type strain, contributes about 75% to the overall rate of nitrite reduction. It is a flavoprotein with a single type of polypeptide, *M*_r 88 kDal (Coleman *et al.*, 1978; Jackson *et al.*, 1981). The prosthetic groups are a non-haem iron–sulphur cluster, sirohaem and loosely-bound FAD (Jackson *et al.*, 1981). Synthesis of this NADH-dependent enzyme, together with the membrane-bound formate–nitrite oxidoreductase which contributes about 20% to the overall rate of nitrite reduction (Abou-Jaoudé *et al.*, 1977, 1979*a*), is repressed during aerobic growth. The third, NADPH-dependent nitrite reductase (EC 1.8.1.2), which functions physiologically as a sulphite reductase, is synthesized under both aerobic and anaerobic conditions, but is regulated by cysteine repression (Kemp *et al.*, 1963).

In contrast to the progress that has been made in understanding the biochemistry of nitrite reduction, there is considerable confusion about the identity of structural and regulatory genes involved in the process. In previous reports from this laboratory we have described *nirA* mutants (also called *fnr* and *nirR* mutants: Cole & Ward, 1973; Lambden & Guest, 1976; Chippaux *et al.*, 1978) which are pleiotropically defective in both of the major pathways for nitrite reduction as well as in the synthesis of other anaerobically-induced reductases. The product of the *nirA*⁺ (*fnr*⁺) gene is a positive control protein which is required for transcription of many of the genes involved in anaerobic redox processes. We subsequently characterized a second group of mutations which mapped in the minute 74 region of the *E. coli* chromosome (Cole *et al.*, 1980).

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All except one of these mutants were pleiotropically defective in the NADH-dependent nitrite reductase and the NADPH-dependent sulphite reductase: their Cys⁻ Nir⁻ phenotype and the mapping data suggested that they were *cysG* mutants defective in the synthesis of sirohaem, a prosthetic group of both of these enzymes.

Amongst the previously characterized *cysG* mutants was a single Cys⁺ Nir⁻ strain [formerly called CB203 but now designated JCB203 to avoid confusion with similarly-designated strains from the collection of Dr Chippaux, (CNRS, Marseille, France)] which appeared to be deficient only in the NADH-dependent nitrite reductase activity. As the mutation in this strain mapped extremely close to the *cysG* gene, the possibility was considered that JCB203 was only partially defective in synthesis of the *cysG* product in such a way that sufficient sirohaem was synthesized to restore the Cys⁺ phenotype, but insufficient to meet the increased demands for nitrite reduction during anaerobic growth (Cole *et al.*, 1980). Other possibilities recognized were that strain JCB203 is defective in an independent gene for nitrite reduction which fortuitously maps close to *cysG*, or is part of the *cysG* transcriptional unit. The product of such an independent gene could be the apoprotein for the NADH-dependent nitrite reductase or, alternatively, it could convert sirohaem to a modified form that is the prosthetic group of nitrite reductase. The primary aims of the experiments described in this paper were to determine whether there is a second gene in the 74 min region of the *E. coli* chromosome which is involved only in nitrite reduction and, if so, to characterize mutants in which it is defective.

Abou-Jaoudé *et al.* (1978*a, b*) have described a range of mutants which appeared to be defective in NADH-dependent nitrite reductase activity. A further aim was to characterize biochemically the Nir⁻ Cys⁺ mutants isolated in the two laboratories and to locate the structural gene for the NADH-dependent nitrite reductase apoprotein.

METHODS

Bacteria and media. Bacterial strains used in this study are listed in Table 1. *E. coli* cultures were derivatives of the K12 strain. Media components were from Oxoid or from Lab M. Glycerol-fumarate plates (Lambden & Guest, 1976) containing 1 g Casamino acids l⁻¹ were incubated anaerobically in a Brewer's jar. Other media were either prepared according to the supplier's instructions, or have been described previously (Cole *et al.*, 1974; Newman & Cole, 1977).

Growth conditions, preparation of extracts and enzyme assays. These were as described by Cole *et al.* (1974) and by Newman & Cole (1978) but with the following modifications. The concentration of nitrite during growth of mutants in 2 litre conical flasks was decreased from 5 mM to 2.5 mM, and 0.4% (w/v) maltose replaced glucose where noted. Washed bacteria were resuspended in 50 mM Tris/HCl, 5 mM-ascorbate, 5 mM-EDTA, pH 8.0, before they were broken in a French pressure cell. Protein concentrations were determined by the microtannin procedure (Mejbaum-Katzenellenboger & Dobryszczyka, 1959). The oxidant for the NAD⁺-activated 'diaphorase' activity associated with NADH-nitrite oxidoreductase apoprotein was 0.1 mM-horse heart cytochrome *c* (Sigma).

Inverted Durham tubes were used to monitor formate hydrogenlyase activity during anaerobic growth in rich media with 0.4% (w/v) glucose as the fermentable substrate. Both Lennox broth (Miller, 1972) and nutrient broth were used interchangeably for these tests. Cysteine (10 µg ml⁻¹) was added to cultures of cysteine auxotrophs.

Transposon Tn10 mutagenesis. The source of transposon Tn10 was fresh stocks of bacteriophage λ NK 370 (Table 1). These were generated by mixing three to five small, freshly-generated plaques with 0.2 ml of an overnight culture of any suppressor-negative strain of *E. coli* (AB2847 or HfrH was normally used) in λ broth (10 g tryptone, 2.5 g NaCl l⁻¹). After 20 min at room temperature, 7.5 ml molten λ top agar (λ broth plus 6.5 g agar l⁻¹) was added and the mixture was distributed to three λ plates (λ broth plus 10 g agar l⁻¹). After about 10 (but less than 12) h at 37 °C, the phage was harvested by transferring the top agar to 2 ml SM (20 mM-Tris/HCl pH 7.5, 0.1 M-NaCl, 10 mM-MgSO₄), homogenizing the suspension with 1 ml CHCl₃ and centrifuging at 5000 *g* for 10 min.

To induce Tn10 mutations, 2 ml cultures of the desired host were grown with aeration to mid-exponential phase in λym broth (λ broth plus 0.2%, w/v, maltose and 0.01%, w/v, yeast extract), harvested by centrifugation and resuspended in 0.1 ml λym broth. The λ suspension was diluted 10-fold and 0.1 ml of undiluted and diluted phage were added to separate tubes of recipient bacteria. After 1 h at 37 °C, the contents of each tube were spread onto a Lennox agar plate containing 15 µg tetracycline ml⁻¹ and 2.5 mM-sodium pyrophosphate. Tetracycline-resistant colonies formed after 24 h at 37 °C.

It was essential to prepare all media within 24 h of use, to generate stocks from fresh plaques and to use the stocks on the day they were prepared. For this reason, λ stocks were not titred before use. Nevertheless, results were extremely variable and few, if any, tetracycline-resistant colonies were obtained from about half of the infection experiments.

Table 1. *Strains used and their source*

Strain	Genotype	Source* or reference
AB312	<i>thr-1 leu-6 thi-1 lacZ4 rpsL8 supE44</i> -Hfr point of origin 12	CGSC
AB2847	<i>aroB mala</i>	CGSC
CGSC4248	<i>argG6 metB1 his-1 leu-6 recA1 mt1-2 xyl-7 malA1 gal-6 lacY1 rpsL104 tonA2 tsx-1 supE44</i>	Acridine orange curing of CGSC4248 F'141
CGSC4315	Prototrophic Hfr <i>malB supE</i>	CGSC
CGSC4248F'141	CGSC4248/F' <i>argG⁺ rpsL⁺ aroB⁺ malA⁺</i>	CGSC
DB6659	<i>srl-300::Tn10 recA56 ilv-318 thr-300 thi-1 spe-300 rel-1</i>	Laboratory stocks
HfrH	Prototroph	Laboratory stocks
JCB203†	<i>nir-203</i> (now <i>nirB203</i>)	Cole <i>et al.</i> (1980)
JCB312†	AB2847 Mal ⁺ <i>cysG72</i>	P1 transduction; JM72 as donor and AB2847 as recipient
JCB401†	CGSC4315 <i>chl-401</i>	CGSC4315 spontaneously resistant anaerobically to 10 mM-chlorate
JCB406	JCB401 <i>cysG406::Tn5</i>	Tn5 mutagenesis with F'141::Tn5
JCB407	JCB401 <i>cysG407::Tn5</i>	Tn5 mutagenesis with F'141::Tn5
JM72	<i>cysG72 thr leu pro his argC thi lac gal mal xyl rpsL</i>	M. C. Jones-Mortimer
LCB82†	<i>nirD82 thr-1 leu-6 lacY1 tonA22 thi-1 ana-1 rpsL</i>	M. Chippaux (Abou-Jaoudé <i>et al.</i> , 1979 <i>b</i>)
LCB84†	As LCB82, but <i>nirF</i>	
LCB85†	As LCB82, but <i>nirE</i>	
LCB190†	As LCB82, but <i>nirG</i>	
LCB197†	As LCB82, but <i>nirH</i>	M. Chippaux (Pascal <i>et al.</i> , 1981)
LCB900	As LCB82, but <i>nir⁺</i> parent	M. Chippaux (Abou-Jaoudé <i>et al.</i> , 1978 <i>b</i>)
MAL103	Mu <i>cts d1</i> (Ap ^R <i>lac</i>) <i>araB::Mu cts argD139(proAB lacIPOZYA) rpsL</i>	M. Casadaban
OR75Ch15	Prototrophic Hfr	Cole <i>et al.</i> (1980)
RVCh11	Prototrophic F ⁻	Strain RV spontaneously resistant to 10 mM- chlorate during anaerobic growth
W3102	<i>cysG3102</i>	M. G. Marinus

* CGSC strains received from B. Bachmann, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn., USA. Other addresses are: M. C. Jones-Mortimer, Department of Biochemistry, Cambridge University, UK; M. Chippaux, Laboratoire de Chimie Bacterienne, CNRS, Marseille, France; M. Casadaban, Department of Biophysics and Theoretical Biology, University of Chicago, Ill., USA; M. G. Marinus, Department of Pharmacology, University of Massachusetts Medical School, Worcester, Mass., USA.

† Strains isolated in different laboratories (including nitrite reductase-deficient mutants) were formerly designated CB; to minimize confusion, extra letters have been added to these strain designations by agreement between the two laboratories.

Mutagenesis with the Casadaban phage Mu d1(Ap^R *lac*). The source of bacteriophage Mu d1(Ap^R *lac*) was a mixed lysate of Mu *cts* and Mu d1(Ap^R *lac*) generated from the double lysogen MAL103. It was used as described by Casadaban & Cohen (1979). After infection of the parent strain, 0.3 ml cultures were incubated for 1 to 2 h at 30 °C with 2 ml Lennox broth to allow expression of ampicillin resistance and plated onto nutrient agar supplemented with 25 µg ampicillin ml⁻¹. Colonies appeared after 36 h at 30 °C.

Temperature stabilization of Mu d1(Ap^R *lac*) *fusion strains*. Ampicillin-resistant mutants were grown to saturation at 30 °C in 1 ml Lennox broth and diluted 50-fold into 5 ml fresh Lennox broth. After 2 h at 30 °C, the cultures were aerated at 42 °C for 20 min and grown to saturation at 37 °C. Serial dilutions were then plated onto nutrient agar and incubated at 37 °C. Colonies from suitable plates were replica plated onto selective media to screen for loss of markers of interest.

Other genetic methods. Nitrosoguanidine mutagenesis and the isolation of mutants defective in anaerobic growth with nitrite as sole nitrogen source were described by Cole & Ward (1973). Two cycles of penicillin enrichment were used before survivors of mutagenesis were tested for their ability to reduce nitrite. Random Tn10- or Mu d1(Ap^R *lac*)-induced mutants were screened for ability to reduce nitrite by the spot test procedure without enrichment (Cole & Ward, 1973).

Table 2. *Characterization of NirD⁻, NirE⁻, NirF⁻, NirG⁻ and NirH⁻ strains*

Strain	Genotype	Nitrite reduction by bacterial suspensions [nmol nitrite reduced min ⁻¹ (mg bacterial dry wt) ⁻¹]		NADH-dependent nitrite reductase [nmol NADH oxidized min ⁻¹ (mg protein) ⁻¹]	Cytochrome <i>c</i> ₅₅₂ content [pmol (mg protein) ⁻¹]
		Glucose- dependent	Formate- dependent		
LCB900	<i>nir</i> ⁺ parent	48	19	166	76
LCB82	<i>nirD</i>	32	19	<10	174
LCB84	<i>nirF</i>	15	6	774	94
LCB85	<i>nirE</i>	18	10	225	66
LCB190	<i>nirG</i>	33	15	403	109
LCB197	<i>nirH</i>	74	<2	1530	169

Transposon Tn5 was introduced into a derivative of the Hfr strain CGSC4315 on the plasmid F'141. After mating, bacteria were harvested by centrifugation, resuspended in Lennox broth plus 0.4 % (w/v) glucose and incubated for 30 min with aeration at 37 °C to ensure expression of kanamycin resistance. Kanamycin was then added to 25 µg ml⁻¹ and cultures were grown to saturation to ensure loss of the F' plasmid due to incompatibility with the F factor resident in the chromosome. Strains JCB406 and JCB407 are independently isolated Kan^R Cys⁻ Nir⁻ derivatives which were purified by single colony isolation.

Bacteriophage P1kc was used for transduction experiments (Miller, 1972).

The *recA* mutation was transferred to F⁻ strains by mating them with the Hfr strain DB6659 which carries a *recA* allele and Tn10 inserted into the nearby *srl* gene (Bachmann, 1983). Tetracycline-resistant colonies were selected and screened for increased sensitivity to UV light (Miller, 1972).

RESULTS AND DISCUSSION

All of the nitrite reductase-deficient mutants previously isolated in this laboratory were, with one exception, also pleiotropically defective for one or more other reductases (Cole *et al.*, 1980). In contrast, Abou-Jaoudé *et al.* (1978, 1979*b*) described mutants with lesions in *nirD*, *E*, *F*, *G* and *H* which apparently were specifically deficient in nitrite reduction. Dr Marc Chippaux kindly provided us with a representative mutant from each group for more detailed biochemical characterization.

After overnight growth in media supplemented with nitrite, suspensions of the *nirD* strain LCB82 reduced nitrite when formate was the electron donor (Table 2). The glucose-dependent rate of nitrite reduction was less than that of the parental strain and no NADH-dependent nitrite reductase activity was detected in bacterial extracts (Table 2). In contrast to the *nirD* mutant, the *nirH* strain LCB197 reduced nitrite rapidly with glucose as the electron donor, but formate-dependent nitrite reductase was inactive. The specific activity of the NADH-dependent nitrite reductase in the *nirH* mutant was higher than in any other strain we have tested and was approximately twice that of the chlorate-resistant mutants which are constitutive for nitrite reductase synthesis during anaerobic growth (compare Table 2 with Jackson *et al.*, 1981). Furthermore, the 88 kDal apoprotein of nitrite reductase was readily detected in unfractionated extracts of this mutant by SDS-PAGE (data not shown). Very little formate-dependent nitrite reductase activity was detected with the *nirH* mutant, but contrary to the earlier reports (Abou-Jaoudé *et al.*, 1979*b*; Pascal *et al.*, 1981), cytochrome *c*₅₅₂ was readily detected in bacterial extracts (Fig. 1). We conclude that the *nirH* gene is required for electron transfer from formate to nitrite rather than for NADH-dependent nitrite reduction.

Formate was an effective electron donor for nitrite reduction by suspensions of strains LCB85 (*nirE*), LCB84 (*nirF*) and LCB190 (*nirG*). As the NADH-dependent nitrite reductase was also very active in these strains, we conclude that they are defective in anaerobic glucose metabolism rather than specifically in nitrite reduction. This conclusion is consistent with the lower rate of glucose-dependent nitrite reduction by suspensions of these mutants than by the *nirH* mutant (Table 2).

Fig. 1. Difference spectrum showing the presence of cytochrome c_{552} in soluble proteins from the *nirH* mutant LCB197. Sodium dithionite was added to reduce proteins in the sample cuvette. The reference sample was oxidized with a few grains of potassium ferricyanide. The spectrum was recorded at room temperature and the protein concentration was 16.5 mg ml^{-1} .

In summary, only two of the mutant classes described by Abou-Jaoudé *et al.* (1978*a*) were deficient in their ability to reduce nitrite and only the *nirD* strain was defective in NADH-dependent nitrite reductase activity. The phenotype of LCB82 was identical to that of strain JCB203, but although both of these lesions were reported to map in the *crp-cysG* region of the *E. coli* chromosome, data for co-transduction with *aroB*⁺ suggest that they might be defective in different genes (compare Cole *et al.*, 1980, with Abou-Jaoudé *et al.*, 1979*b*). To resolve this point a systematic search was made for other mutants specifically deficient in NADH-dependent nitrite reductase activity.

Isolation of mutants defective only in NADH-dependent nitrite reduction

Three mutagenesis techniques and five different parental strains were used to generate a wide range of nitrite reductase deficient mutants. The parents included the *nirH* mutant LCB197 and two chlorate-resistant mutants, all of which synthesize high activities of NADH-dependent nitrite reductase but cannot use formate to reduce nitrite. Two Hfr strains were also used to facilitate transfer of interesting mutations to different genetic backgrounds. Mutants defective in nitrite reduction were purified by single colony isolation, tested for formate hydrogenlyase activity and used as donors in bacteriophage P1-mediated transduction with the *aroB* mutant AB2847 or its derivative AB2847 Arg⁻: μ_{is} as recipient (Table 3). Only one of 25 new mutants, JCB313, was Cys⁻. The 39% co-transduction of both the Nir⁻ and the Cys⁻ phenotypes with Aro⁺ indicated that JCB313 carries a *cysG*::Mu d1(Ap^R *lac*) insertion.

The remaining mutants fell into two groups which we provisionally designated *nirA* (*fnr*) or *nirB* (Table 3). All six Tn10 mutants were deficient in formate hydrogenlyase activity and were unable to grow anaerobically on glycerol-fumarate plates. The tetracycline-resistance determinant was not co-transducible with *aroB*⁺ (Table 3) or with *cysG*⁺. We conclude that these are *nirA* (*fnr*) mutants. The most interesting aspect of these experiments was our failure to generate Tn10 insertion mutations in either the *cysG* or the *nirB* genes.

In contrast to Tn10 mutagenesis, nitrosoguanidine and Mu d1(Ap^R *lac*) mutagenesis resulted in the isolation of a series of mutants defective only in NADH-dependent nitrite reductase activity. The retention of formate hydrogenlyase activity, the Cys⁺ phenotype, the ability to grow anaerobically on glycerol-fumarate plates and the 15 to 45% co-transduction of the nitrite reduction defects with *aroB*⁺ were consistent with these strains being defective in a nitrite

Table 3. *Recently-isolated mutants deficient in NADH-dependent nitrite reductase activity*

Mutant	Parent	Mutagen	Formate hydrogenlyase*	Other relevant information	Suggested genotype
JCB301	RVChl1	Mu d1(Ap ^R lac)	—	Nir ⁻ Amp ^R 20% co-transducible with <i>aroB</i> ⁺	<i>nirB</i>
JCB302	RVChl1	Mu d1(Ap ^R lac)	—	Derivative isolated with deletion into <i>cysG</i> (see later)	<i>nirB</i>
JCB303	RVChl1	Mu d1(Ap ^R lac)	—	As JCB302	<i>nirB</i>
JCB304	RVChl1	Mu d1(Ap ^R lac)	—	Nir ⁻ Amp ^R co-transducible with <i>aroB</i> ⁺	<i>nirB</i>
JCB305	RVChl1	Mu d1(Ap ^R lac)	—	As JCB304	<i>nirB</i>
JCB306	RVChl1	Mu d1(Ap ^R lac)	—	As JCB304	<i>nirB</i>
JCB307	OR75Ch15	NG	—	Nir ⁻ >90% co-transducible with <i>cysG</i> ⁺ ; 45% co-transducible with <i>aroB</i> ⁺	<i>nirB</i>
JCB308	OR75Ch15	NG	—	Nir ⁻ not co-transducible with <i>aroB</i> ⁺ ; defective cytochrome <i>c</i> ₅₅₂ synthesis	<i>nirA</i>
JCB309	HfrH	NG	+	Nir ⁻ >90% co-transducible with <i>cysG</i> ⁺ ; 24% co-transducible with <i>aroB</i> ⁺	<i>nirB</i>
JCB310	HfrH	NG	+	Nir ⁻ >90% co-transducible with <i>cysG</i> ⁺ ; 28% co-transducible with <i>aroB</i> ⁺	<i>nirB</i>
JCB311	HfrH	Mu d1(Ap ^R lac)	+	Amp ^R co-transducible with <i>aroB</i> ⁺	<i>nirB</i>
JCB313	HfrH	Mu d1(Ap ^R lac)	+	Also Cys ⁻ ; Amp ^R Cys ⁻ 100% co-transducible with Nir ⁻ ; 39% with <i>aroB</i> ⁺	<i>cysG</i>
JCB315	LCB197 <i>nirH</i>	Tn10	—	Nir ⁻ phenotype complemented by the <i>fnr</i> ⁺ plasmid pCH21	<i>nirA</i>
JCB316	LCB197 <i>nirH</i>	Tn10	—	As JCB315	<i>nirA</i>
JCB317	LCB197 <i>nirH</i>	Tn10	—	—	<i>nirA</i>
JCB318	LCB197 <i>nirH</i>	Tn10	—	—	<i>nirA</i>
JCB319	LCB197 <i>nirH</i>	Tn10	—	—	<i>nirA</i>
JCB220	LCB197 <i>nirH</i>	Tn10	—	Tet ^R Nir ⁻ phenotype not co-transducible with <i>aroB</i> ⁺	<i>nirA</i>
JCB382	AB312	Mu d1(Ap ^R lac)	+	—	<i>nirB</i>
JCB383	AB312	Mu d1(Ap ^R lac)	+	Derivative isolated with deletion extending into <i>cysG</i>	<i>nirB</i>
JCB384	AB312	Mu d1(Ap ^R lac)	+	As JCB383	<i>nirB</i>
JCB385	AB312	Mu d1(Ap ^R lac)	+	Amp ^R co-transducible with <i>aroB</i> ⁺	<i>nirB</i>
JCB386	AB312	Mu d1(Ap ^R lac)	+	As JCB385	<i>nirB</i>
JCB387	AB312	Mu d1(Ap ^R lac)	+	As JCB385	<i>nirB</i>
JCB388	AB312	Mu d1(Ap ^R lac)	+	As JCB385	<i>nirB</i>

* Formate hydrogenlyase is inactive in chlorate-resistant strains due to loss of normal molybdenum incorporation into the formate dehydrogenase component. This test is therefore useful only for mutants derived from chlorate-sensitive strains.

NG, Nitrosoguanidine.

Table 4. *Temperature-resistant derivatives of Mu d1(Ap^R lac)*

Original temperature-sensitive mutant	Phenotype of temperature-sensitive parent	Phenotype of temperature-resistant derivative
JCB301	Ap ^R Lac ⁺ Nir ⁻ Cys ⁺	Ap ^S Lac ⁻ Nir ⁻ Cys ⁺
JCB302	Ap ^R Lac ⁺ Nir ⁻ Cys ⁺	Ap ^S Lac ⁺ Nir ⁻ Cys ⁻
JCB303	Ap ^R Lac ⁺ Nir ⁻ Cys ⁺	Ap ^S Lac ⁻ Nir ⁻ Cys ⁺
		Ap ^S Lac ⁻ Nir ⁻ Cys ⁻
JCB383	Ap ^R Lac ⁻ Nir ⁻ Cys ⁺	Ap ^S Lac ⁻ Nir ⁻ Cys ⁺
		Ap ^S Lac ⁻ Nir ⁻ Cys ⁻

reductase gene other than *cysG* but located in the 74 min region of the chromosome. The phenotypes of these mutants were similar to that of the previously described strains JCB203 and LCB82.

Biochemical characterization of mutants deficient in NADH-dependent nitrite reductase activity

All of the mutants tentatively designated *nirB*, together with their parental strains, were grown anaerobically in rich media supplemented with glucose and nitrite. Glucose-dependent and formate-dependent rates of nitrite reduction by bacterial suspensions and NADH-dependent nitrite reductase activities of extracts were determined. The sirohaem-deficient apoprotein of purified NADH-nitrite oxidoreductase can still catalyse the reduction of mammalian cytochrome *c* by NADH and a characteristic feature of this activity is its activation by NAD⁺. Mutants defective in the synthesis of the sirohaem prosthetic group should retain this activity, but absence of an NADH⁺-activated cytochrome *c* reductase should be a diagnostic characteristic of strains unable to synthesize the nitrite reductase apoprotein; this activity was also determined.

All of the newly-isolated *nirB* mutants reduced nitrite more slowly than the parental strain with glucose as the electron donor, but nitrite reduction by formate was unaffected or, if anything, slightly more rapid. Neither NADH-dependent nitrite oxidoreductase nor NAD⁺-activated cytochrome *c* reductase activity was detected in any of these mutants or in the *nirD* strain LCB82. Surprisingly, an NAD⁺-activated cytochrome *c* reductase was readily detected in the previously-described mutant JCB203: this result would be consistent with *nirB* being the structural gene for the NADH-nitrite oxidoreductase apoprotein if the *nirB203* protein retains normal binding domains for NADH, NAD⁺ and cytochrome *c* but lacks the sirohaem or nitrite-binding domains (Jackson *et al.*, 1982).

All of the *nirB* mutants grew well on minimal agar unsupplemented with cysteine, indicating that the *cysG*⁺ gene was intact and expressed.

Temperature stabilization of the Mu d1(Ap^R lac) fusion strains

Ampicillin-sensitive derivatives of many of the operon fusion mutants were isolated after heat induction. Some of these temperature-resistant derivatives had also lost either the ability to grow without cysteine, or the Lac⁺ phenotype controlled by the *nirB* promoter, or both (Table 4). The alteration of three or four of these phenotypes (including temperature-sensitivity) by a single event was assumed to be caused by a deletion of chromosomal DNA during aberrant excision of the Mu d1(Ap^R lac) prophage. Loss of only one or two characteristics could result from either a transpositional deletion or inversion event. The phenotypes of the various derivatives are consistent with *nirB* and *cysG* being independent genes located close to each other on the *E. coli* chromosome. They establish that no essential genes or biosynthetic determinants are located between *cysG* and *nirB*, but do not establish whether the two genes are contiguous or are expressed independently.

Location of the nirB gene by transduction

All of the *nirB* mutations together with several of the deletion mutations in temperature-resistant derivatives of *nirB*:Mu d1(Ap^R lac) strains were transferred to the Aro⁻ mutant AB2847. The Arg⁻ derivative of AB2847 carrying a Mu₁₈ prophage was the recipient for Mu d1(Ap^R lac) insertion mutations.

In each experiment, between 15% and 45% of the Aro⁺ transductants were Nir⁻. When ampicillin-resistant transductants were selected, all were Nir⁻ but at most 20% were Aro⁺. The presence of the Mu d1(Ap^R lac) prophage in these donor strains decreased the apparent co-transduction frequency, so map distances cannot be calculated from such data.

Three of the *nirB* mutations, the *nirD* mutation in strain LCB82 and four *cysG* mutations were transferred to AB2847 and over 100 Aro⁺ transductants were scored for their Nir, Cys, Mal and, where appropriate, kanamycin resistance phenotypes. As previously reported by Cole *et al.* (1980), the Cys⁻ and Nir⁻ phenotypes of *cysG* strains were 100% cotransducible. This provided further evidence that the Cys⁻ and Nir⁻ phenotypes are due to a single mutation and that Tn5 is

Table 5. *Cotransduction frequencies for the transfer of Nir⁻ mutations with aroB⁺*

Donor		No. of transductants with the phenotype:				Cotransduction frequency (%) Aro ⁺ Nir ⁻ /Aro ⁺	Designation of Aro ⁺ Mal ⁻ Nir ⁻ derivative
Strain	Genotype	Aro ⁺	Aro ⁺ Nir ⁻	Aro ⁺ Mal ⁺	Aro ⁺ Nir ⁻ Mal ⁺		
JCB406	<i>cysG406</i>	108	36	30	0	33	JCB421
		108	36	—	—	33	—
JCB407	<i>cysG407</i>	108	41	30	3	38	JCB422
		108	30	—	—	28	—
W3102	<i>cysG3102</i>	108	7	44	1	7	JCB423
		108	17	—	—	16	—
JM72	<i>cysG72</i>	108	21	44	6	22	JCB424
		105	32	—	—	31	—
JCB309	<i>nirB309</i>	100	24	58	10	24	JCB425
		103	35	—	—	34	—
JCB307	<i>nirB307</i>	105	24	64	5	30	JCB426
		107	34	—	—	32	—
JCB203	<i>nirB203</i>	108	25	56	7	23	JCB427
		108	40	—	—	37	—
LCB82	<i>nirD82</i>	108	21	—	0	20	JCB428

Table 6. *Transduction data for determining the relative position of cysG72 and the nir mutations*

Strain CB312 (*cysG72 aroB*) was transduced to Cys⁺ using bacteriophage P1 which had been propagated on the donor strains. After purification transductants were screened for their Aro⁺ phenotype by replica plating, and for their Nir⁺ phenotype by the spot testing procedure.

Donor		No. of transductants with the phenotype:				Recombination frequency (%) Cys ⁺ Nir ⁺ /Cys ⁺	Map distance (min)
Strain	Genotype	Cys ⁺	Cys ⁺ Nir ⁺	Cys ⁺ Nir ⁺ Aro ⁻	Cys ⁺ Aro ⁺		
CB425	<i>nirB309</i>	141	16	5	95	11.8	0.078
CB426	<i>nirB307</i>	144	15	7	91	10.4	0.072
CB427	<i>nirB203</i>	119	5	2	101	4.2	0.028
CB428	<i>nirD82</i>	108	17	6	94	12.3	0.086

inserted in the *cysG* gene in strains W3102, JCB406 and JCB407. All of the *nirB* and the *cysG* mutations were 16 to 38% co-transducible with *aroB⁺* and few, if any, Aro⁺ Mal⁺ transductants were also Nir⁻ (Table 5). This indicates that each of these Nir⁻ mutations is located on the opposite side of *aroB* to *malA* at about minute 73.5 on the *E. coli* linkage map (Wu, 1966; Bachmann, 1983).

From each transduction an Aro⁺ Mal⁻ Nir⁻ (Cys⁻) colony was purified for biochemical characterization and for use in strain constructions for complementation analysis. These strains were designated JCB421 to JCB428.

The relative order of the eight Nir⁻ mutations used for the previous experiments was determined by three-point crosses in which an *aroB cysG72* strain, JCB312, was transduced to Cys⁺ with phage which had been propagated on the Nir⁻ strains JCB421 to JCB423 and JCB425 to JCB428. Both Cys⁺ Nir⁺ Aro⁺ and Cys⁺ Nir⁻ Aro⁻ transductants were obtained when a *nirB* or the *nirD* strain was the donor, indicating that the gene order is *nirB(D)-cysG72-aroB* (Table 6). A tentative genetic map has been constructed based on the recombination frequencies between the donor Nir⁻ and the *cysG72* mutations (Fig. 2).

A very low frequency of recombination was also detected when any of the three *cysG::Tn5* strains was the donor and the *cysG72* strain was the recipient (Table 7). All of the Cys⁺ colonies were Nir⁺. All of the Cys⁺ transductants were also Aro⁻, so each of the *cysG::Tn5* mutations is located between the *cysG72* and *aroB* mutations. In these experiments, the number of Aro⁺ transductants was also determined so that the recombination frequency between the donor and recipient Cys⁻ mutation could be calculated relative to an internal control. In each case the very low recombination frequency implied a map distance of less than 0.01 min. The only conclusions

Fig. 2. Linkage map of the Nir⁻ mutations located around minute 74 of the *E. coli* chromosome. The positions of individual mutations were determined relative to the *cysG72* allele by three-point transductional crosses. The relative positions with respect to other mutations have not been determined, but the map illustrates the arrangement of the *cysG* : Tn5, *nirB* and '*nirD82*' mutations relative to *cysG72*.

Table 7. Transduction data for determining the relative positions of *cysG72* and the *cys* mutations

Strain CB312 (*cysG72 aroB*) was transduced to Cys⁺ or Aro⁺ using bacteriophage P1 which had been propagated on the donor strains. Cys⁺ transductants were purified and their Aro phenotype was determined by replica plating.

Donor		No. of transductants with the phenotype:			Recombination frequency (%) Cys ⁺ /Aro ⁺	Map distance† (min)
Strain	Genotype	Cys ⁺	Cys ⁺ Aro ⁻	Aro ⁺⁺		
CB421	<i>cysG406</i>	93	93	10280	0.9	0.006
CB422	<i>cysG407</i>	59	59	4770	1.2	0.008
CB423	<i>cysG3102</i>	59	59	4910	1.2	0.008

* Aro⁺ was selected independently of the Cys⁺ selection.

† Subject to large error (see text).

drawn from these results are that the four Cys⁻ Nir⁻ mutations are extremely close together and that mutations which produce the Nir⁻ Cys⁺ and Nir⁻ Cys⁻ phenotypes are arranged in two clusters. This clustering of mutations which cause similar phenotypes suggests the existence of at least two genes and supports the *cysG* and *nirB* designations that were previously made on the basis of phenotype.

The phenotypes caused by the various *nirB* and *cysG* mutations were confirmed by determining the rate of nitrite reduction by formate, the NADH-nitrite oxidoreductase activity and the concentration of cytochrome *c*₅₅₂ in cell extracts of the isogenic strains JCB421 to JCB427 (Table 8). The *nirB* and the *nirD82* mutations resulted in total loss of NADH-dependent nitrite reductase activity but nitrite reduction by formate and synthesis of cytochrome *c*₅₅₂ were essentially unaffected. The *cysG* strains were also deficient in NADPH-dependent sulphite reductase activity, as expected (Table 8).

Complementation analysis of the *nirB*, *nirD* and *cysG* loci

A set of spontaneously arising F' plasmids carrying *cysG*, *nirB* or the *nirD82* mutation was constructed by the procedures summarized in Table 9. These plasmids were then transferred by conjugation into *nirB recA* or *cysG recA* recipients. Mal⁺ merodiploids were purified by single colony isolation and tested for their ability to reduce nitrite after overnight growth with maltose and nitrite. When the F' plasmid carried transposon Tn5, kanamycin was also included in the growth medium to select for retention of the plasmid.

Table 8. *Biochemical characteristics of strain AB2847 and derivatives carrying cysG or nirB mutations*

Sulphite reductase activities were determined with bacteria which had been grown aerobically in minimal medium containing L-djenkolic acid as the sole sulphur source. Bacteria for other assays were grown anaerobically in 2 litres of half-strength nutrient broth in minimal salts supplemented with glucose and nitrite, as described in Methods.

Strain and genotype	NADH-dependent nitrite reductase [nmol NADH oxidized min ⁻¹ (mg protein) ⁻¹]	NADPH-dependent sulphite reductase [nmol NADPH oxidized min ⁻¹ (mg protein) ⁻¹]	Formate-dependent nitrite reductase [nmol NO ₂ ⁻ reduced min ⁻¹ (mg dry weight) ⁻¹]	Cytochrome <i>c</i> ₅₅₂ content [pmol (mg protein) ⁻¹]
AB2847 <i>nirB</i> ⁺ <i>cysG</i> ⁺	180	29	16	69
JCB421 <i>nirB</i> ⁺ <i>cysG</i>	<10	<1	25	40
JCB422 <i>nirB</i> ⁺ <i>cysG</i>	<10	<1	22	68
JCB423 <i>nirB</i> ⁺ <i>cysG</i>	<10	<1	20	43
JCB424 <i>nirB</i> ⁺ <i>cysG</i>	<10	<1	31	62
JCB425 <i>nirB</i> <i>cysG</i> ⁺	<10	20	17	132
JCB426 <i>nirB</i> <i>cysG</i> ⁺	<10	32	36	134
JCB427 <i>nirB</i> <i>cysG</i> ⁺	<10	23	20	35

Table 9. *Construction of F' plasmids carrying nirB or cysG mutations*

Aim of construction	Donor	Recipient	Select	Strain purified
Transfer a <i>cysG</i> mutation to the Hfr strain AB312 by transduction	JCB423	AB312	Kan ^R	Aro ⁺ Cys ⁻ Kan ^R = JCB443 Hfr <i>cysG3102::Tn5</i>
Cotransduce <i>aroB</i> with <i>cysG</i> ⁺ into the Hfr <i>cysG</i> strain	AB2847	JCB443	Cys ⁺	Cys ⁺ Mal ⁺ Aro ⁻ = JCB440 Hfr <i>cysG</i> ⁺ <i>aroB</i>
Cotransduce other <i>cysG</i> mutations into the Hfr Cys ⁺ Aro ⁻ strain with <i>aroB</i> ⁺	JCB421, 422, 424	JCB440	Aro ⁺	Aro ⁺ Cys ⁻ Nir ⁻ = JCB441, 442 and 444 Hfr <i>aroB</i> ⁺ <i>cysG</i>
Cotransduce <i>nirB</i> mutations into the Hfr Cys ⁺ Aro ⁻ strain with <i>aroB</i> ⁺	JCB425 to JCB428	JCB440	Aro ⁺	Aro ⁺ Nir ⁻ = JCB445 to JCB448 Hfr <i>aroB</i> ⁺ <i>nirB</i>
Transfer spontaneously arising <i>F'aroB</i> ⁺ <i>malA</i> ⁺ <i>cysG</i> <i>argG</i> ⁺ plasmids to a <i>recA</i> recipient	JCB441 to JCB444	CGSC4248	Mal ⁺ Arg ⁺ Thr ⁺ Thi ⁺	JCB450 to JCB453 F ⁻ <i>recA</i> F' <i>malA</i> ⁺ <i>aroB</i> ⁺ <i>cysG</i> <i>argG</i> ⁺
Transfer spontaneously arising F' <i>aroB</i> ⁺ <i>malA</i> ⁺ <i>nirB</i> plasmids to a <i>recA</i> recipient	JCB445 to JCB448	CGSC4248	Mal ⁺ Arg ⁺ Thr ⁺ Thi ⁺	JCB454 to JCB457 F ⁻ <i>recA</i> F' <i>malA</i> ⁺ <i>aroB</i> ⁺ <i>nirB</i> <i>argG</i> ⁺

Table 10. Complementation analysis of the *nirB* and *cysG* loci

Merodiploid strains were grown with maltose as the fermentable carbon source to maintain selection for retention of the F' plasmid and, where appropriate, 20 µg kanamycin ml⁻¹ was also added. Strains JCB431 to JCB437 are *srl::Tn10recA* derivatives of JCB421 to JCB427 (parental strain AB2847; see Tables 5 and 8).

Host	F' plasmid	NADH-dependent nitrite reductase [nmol NADH oxidized min ⁻¹ (mg protein) ⁻¹]	Cytochrome <i>c</i> reductase [nmol cyto <i>c</i> reduced min ⁻¹ (mg protein) ⁻¹]	
			- NAD ⁺	+ NAD ⁺
JCB431 <i>cysG406::Tn5</i>	None	< 10	940	2500
	F' <i>nir</i> ⁺ <i>cys</i> ⁺	370	1610	4160
	F' <i>nirB203</i>	13	1120	2890
	F' <i>nirB130</i>	< 10	134	134
JCB433 <i>cysG3102::Tn5</i>	None	< 10	1220	2260
	F' <i>nir</i> ⁺ <i>cys</i> ⁺	532	ND	ND
	F' <i>nirB203</i>	< 10	ND	ND
	F' <i>nirB130</i>	< 10	ND	ND
JCB435 <i>nirB309</i>	None	< 10	280	220
	F' <i>nir</i> ⁺ <i>cys</i> ⁺	43	345	690
	F' <i>cysG406::Tn5</i>	179	515	1095
	F' <i>cysG3102::Tn5</i>	192	461	1040
	F' <i>cysG72</i>	< 10	122	101
	F' <i>nirB203</i>	< 10	790	1440
JCB437 <i>nirB203</i>	None	< 10	2860	7240
	F' <i>nir</i> ⁺ <i>cys</i> ⁺	187	910	2010
	F' <i>cysG406::Tn5</i>	225	2490	5990
	F' <i>cysG3102::Tn5</i>	296	2320	5880
	F' <i>cysG72</i>	18	818	2370

ND, Not determined.

The wild-type plasmid F'141 complemented the defects in both *cysG* and *nirB* mutants, confirming that both types of mutation are recessive in *trans* (Table 10). The results obtained with F' plasmids carrying mutations were not entirely as expected, because although *cysG::Tn5* plasmids complemented NirB⁻ strains, no complementation was observed with F'*cysG72* (Table 10). Plasmids carrying *nirB* mutations and an intact *cysG* gene restored the Cys⁺ phenotype of *cysG* mutants but the merodiploids were still Nir⁻. This indicates that although the *cysG*⁺ gene on the F' plasmid was expressed, the *nirB*⁺ gene on the chromosome was not.

The F'*cysG::Tn5* plasmids used for the complementation experiments were extremely unstable and it was essential to maintain selective pressure for plasmid retention during overnight growth in 2 litre cultures. Even so, only between 24% and 63% of the bacteria assayed were Mal⁺, and these Mal⁺ colonies generated both Mal⁺ and Mal⁻ colonies when restreaked onto MacConkey-maltose agar. In contrast, the Mal⁺ phenotype of merodiploids carrying the F'*cysG72* plasmid was stably maintained.

The F'*nirB203* plasmid partially restored an NAD⁺-activated cytochrome *c* reductase, but not nitrite reductase, activity to the *nirB* mutant JCB435. This supports the suggestion that nitrite reductase apoprotein, in a form inactive for nitrite reduction, is synthesized by strains carrying the *nirB203* mutation. Furthermore, either the gene coding for this protein or a gene essential for its synthesis is located in the minute 69 to 75 region of the *E. coli* chromosome because the F' plasmid used carries genes from *argG* at minute 69 to *malA* at minute 75.

None of the *nirB* mutations or the *nirD82* mutation was complemented by a *nirB* or the *nirD82* plasmid; similarly, none of the F' plasmids carrying *cysG* mutations complemented either the Cys⁻ or the Nir⁻ phenotypes of chromosomal *cysG* mutations. We therefore conclude that the NirB⁻ and NirD⁻ phenotypes are due to mutations in a single gene, *nirB*, which is close to but independent from the *cysG* gene.

In summary, the data presented in this paper establish that two genes essential for NADH-dependent nitrite reduction are located in the 74 minute region of the *E. coli* chromosome.

Although they map extremely close together, their expression is at least partially independent. The *nirB* gene is located between *crp* and *cysG*. The large number of mutants isolated with defects in *nirB* and our failure to isolate other mutants deficient only in NADH-dependent nitrite oxidoreductase activity with lesions mapping elsewhere on the *E. coli* chromosome strongly suggest that *nirB* is the structural gene for the 88 kDal nitrite reductase apoprotein. The formal possibility remains, however, the *nirB* encodes a positive control protein or some other function that is essential for nitrite reductase synthesis.

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