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Transduction of Nitrate Reductase Loci of *Escherichia coli* by Phages *P1* and λ

W. A. VENABLES
Department of Microbiology, University College of South Wales and Monmouthshire, Cardiff, Wales

J. R. GUEST
Department of Microbiology, University of Sheffield, England

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Summary. A lactate-nitrate medium suitable for genetic studies with nitrate reductase mutants (*nar*⁻) of *Escherichia coli* was devised. This permitted the selection of *nar*⁻ strains by their failure to use nitrate as terminal electron acceptor during anaerobic growth, in addition to the selection procedure based on the chlorate resistance of *nar*⁻ mutants. Transduction studies with phage *P1* and *nar*⁻ mutants from both sources demonstrated the existence of at least three *nar* genes in the *gal* region of the *E. coli* linkage map, their relative positions being: *gal* .... *narF* .... *bio* .... *narD* .... *narE*. Using λ phage cotransduction of *narD* with *bio* was observed and several independently-isolated defective λ-transducing phages were examined. Phage λ also transduced the *narF* gene with *gal* linkage but the *narE* gene was not λ-transducible.

Introduction

Under anaerobic conditions the toxicity of chlorate towards *Escherichia coli* depends on the presence of nitrate reductase. Three classes of chlorate resistant mutant of *Escherichia coli* K12 have recently been described (PUIG and AZOULAY, 1967) and the corresponding genetic loci have been designated *chlA*, *B* and *C*. In addition to their resistance to chlorate all three classes lacked nitrate reductase and two of them (*chlA* and *B*) also lacked formate hydrogenlyase. The *chlA* locus has been mapped between *attλ* and *bio* and *chlB* lies close to *mtl* on the *E. coli* linkage map (PUIG, AZOULAY and PICHINOTY, 1967; TAYLOR and TROTTER, 1967).

In the present studies a lactate-nitrate medium suitable for genetic studies with nitrate reductase-less strains strains was devised.This medium also permitted direct selection of such mutants by virtue of their inability to use nitrate as a terminal electron acceptor during anaerobic growth. Mutants isolated by this procedure were phenotypically similar to *chlA* and *chlB* mutants (VENABLES, WIMPENNY and COLE, 1967, 1968). Genetic studies, primarily intended to compare the genetic identity of these mutants with mutants selected for resistance to chlorate, pointed to the existence of a new locus in the region of *gal* and *bio*. Subsequently, mutants selected for either property were examined in detail and at least 3 new classes of nitrate reductase-less/chlorate resistant mutant (*nar*⁻) have been found in this region of the *E. coli* chromosome.

Materials and Methods

Bacteria and Phages. The following strains of *E. coli* K12 were used: W1485(F^+); W602 (F^-, *leu*⁻, *gal*⁻, *bio*⁻, *str*^{r}, *thi*⁻); AT2687 (F^-, *bio*⁻, *str*^{r}, *met*⁻); PA3476 (F^-, *thr*⁻, *leu*⁻, *lac*⁻, *suc*⁻, *gal*⁻, *bio*⁻, *his*⁻, *str*^{r}, *arg*⁻, *thi*⁻); AB1621 (F^-, *lac*⁻, *gal*⁻, *str*^{r}, *thi*⁻); HfrH (*thi*⁻) and W3101

F_2-*gal* (*F'*, *gal*$^-$/*F-gal*$^+$). Phage *P1kc* was obtained from Dr. C. YANOFSKY and phage *λ*, *λgc* and *λ*-antiserum were kindly provided by Dr. B. A. FRY.

Media. The minimal medium of VOGEL and BONNER (1956) was used with either glucose (0.2%) as carbon source, galactose (0.5%) plus bromothymol blue for *gal*$^+$ selection or acetate (50 mM) for *suc*$^+$ selection. The lactate nitrate medium (LN) devised for genetic studies with nitrate reductase mutants contained (per litre) $NaH_2PO_4 \cdot 2H_2O$, 6.08 g; K_2HPO_4, 10.6 g; $(NH_4)_2SO_4$, 2 g; KNO_3, 4 g; potassium lactate (50%) 7 ml; vitamin-free casamino acids (Difco) 0.4 g and 5 ml of a salts solution which in turn comprised (g/l) $MgSO_4 \cdot 7H_2O$, 10; $MnCl_2 \cdot 4H_2O$, 1; $FeSO_4 \cdot 7H_2O$, 0.05; $CaCl_2$, 0.1 and a trace of conc. HCl to clarify. Minimal media including LN were supplemented with vitamins and amino acids according to the particular requirements of the bacteria to be grown or selected.

The chlorate medium, used mainly for mutant selection consisted of Oxoid Nutrient Broth to which $KClO_4$ (0.1%) and glucose (0.2%) were added. Bacterial stocks were maintained on plates of L-agar (LENNOX, 1955) and the routine liquid growth media were L-broth and *λ*-broth (g/l: Oxoid tryptone, 20; Lab-Lemco, 2 and NaCl, 5; final pH 7.0). Media were solidified with agar 1.0—1.5% for plates and 0.6% for soft overlayers. With *λ* the phage dilution fluid was *λ*-diluent (WEIGLE, MESELSON and PAIGEN, 1959).

Mutant Selection. Mutants were selected from HfrH, AB1621, AT2687 and W602 parents in two ways: a) by their inability to grow anaerobically on LN medium following treatment with *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine (MNNG), penicillin selection and replica plating, or b) by their resistance to chlorate by direct selection anaerobically on chlorate medium, with or without prior exposure to mutagen. Each mutant was given a number prefixed by N or C according to whether it was isolated by method a) or b) respectively. Regardless of the method of selection all the mutants were resistant to chlorate and unable to grow on LN medium. All of the mutants to be considered here lacked nitrate reductase and formate hydrogenlyase and had mutant loci in the *gal*, *bio* region of the linkage map.

Conjugation. Interrupted mating was performed by the method of DE HAAN and GROSS (1962) and conjugation by the cross-streak technique of BERG and CURTISS (1967).

Transduction with Phage P1kc. The media and methods of LENNOX (1955) were employed. Lysates of donor strains were prepared by confluent lysis and phage multiplicities of 2 were used in transduction.

Transduction with Phage λ. Lysates of *λ* phage were prepared by inducing exponential cultures of W1485(*λ*) or other strains lysogenized by *λ*. "Lytic" lysates, used for example as helper phage, were prepared by the confluent lysis overlayer technique and two successive cycles were made initially to prevent carrying-over any transducing phage. In all cases the indicator organism for phage assay was W1485. For transduction, stationary phase *λ*-broth cultures of recipient organisms were normally resuspended in 0.01 M-$MgSO_4$ and mixed with an equal volume of lysate to give a multiplicity of infection of 5. After adsorption at 37° for 20 min, samples containing 0.75—3.0×10^8 bacteria were plated in soft overlayers of the selective medium. Immunity to *λ* was tested by streaking cultures or colonies suspended in *λ*-broth over a line of *λgc* (prepared from a loopful of 2×10^9 phage/ml) and examining the intersection after incubation.

Linkage Analysis. To determine the inheritance of non-selective markers, colonies of the selected types were patched on the same medium (with or without prior purification) and then replicated to appropriate test media. For scoring *bio* markers the first replica plate was re-replicated immediately to ensure the transfer of a small inoculum and a minimum carry-over of biotin.

Detection of Heterogenotes and Assay of Defective Transducing Phage (λdnD). To detect heterogenotes a method based on those of FRASER (1962) and MATSUSHIRO (1963) was devised. Patches of LFT-*narD*$^+$ transductants from *F*$^-$ *nar*$^-$ *met*$^-$ recipients were prepared on LN medium and replicated to plates of *λ*-agar which had been spread with 10^9 *λ* from a "lytic lysate". These plates were incubated for about 5 hr, irradiated, incubated a further 2 hr and the cells killed by inverting over $CHCl_3$. They were then replicated to plates of LN medium containing no methionine (LN-M) which had been spread with 3×10^8 cells of a washed overnight culture of an *F*$^-$*nar*$^-$*met*$^+$ strain. The positions of heterogenotic transductants on the original plates were observed as areas of *nar*$^+$*met*$^+$ growth on the final replica plates after

an incubation period of 44 hr under H_2/CO_2. For this test a *met*$^+$ revertant of AT2876C25 was used as the indicator organism and the casamino acid component of the LN medium was replaced by an artificial mixture of amino acids based on the composition of casein but lacking methionine. Similar results were obtained with a λ-lysogenic indicator strain. Suspected heterogenote patches were purified on λ-agar, re-tested and cultures from active single colonies were used for HFT-lysate production.

The assay procedure for *λdnD* was similar to that for *λdg* (KAISER and HOGNESS, 1960). Samples (0.1 ml) containing *λdnD* diluted in λ-diluent were mixed with 0.05 ml helper λ (1.2×10^{10}/ml) and adsorbed to 0.1 ml of stationary phase AT2687C25 (3×10^9/ml) for 20 min at 37°. Soft LN agar (2.5 ml) was then added and the mixture poured over LN medium and incubated anaerobically for the selection of *nar*$^+$.

Caesium Chloride Centrifugation. The HFT-lysates examined were purified by differential centrifugation and the phage resuspended in λ-diluent. Density gradient centrifugation at 23,000 r.p.m. for 22—36 hr at 4° in the SW39 rotor of a Spinco L2-50 centrifuge was according to WEIGLE *et al.* (1959). From 3 ml samples approximately 100 fractions were collected as drops from the bottom of the centrifuge tube into 1 ml aliquots of λ-diluent (3 drops/fraction).

Results

Transduction with Phage P1

The selection of mutants of *E. coli* lacking nitrate reductase and the selection of wild-type recombinants from these necessitates the use of a medium, growth upon which requires the presence of a functional nitrate reductase system. Since nitrate assimilation by *E. coli* is poor it was necessary to devise conditions in which nitrate reductase fulfils only a respiratory role. This was accomplished by using lactate as a C and energy source, NH_4 and amino acids as sources of N and incubating anaerobically (under H_2 with 5% CO_2) but with nitrate present as terminal electron acceptor. Details of the medium (LN) are given in Materials and Methods and it should be made clear that aerobically wild-type and nitrate reductase mutants grow well on this medium.

Several mutants were selected for their inability to grow anaerobically on LN medium and subsequently many more mutants with the same phenotype were selected by virtue of their resistance to chlorate. The use of the chlorate medium for genetic studies, other than for mutant selection is limited because chlorate resistance is recessive; growth in the absence of chlorate is necessary to permit segregation and phenotypic expression of resistant recombinants. The LN medium therefore possesses considerable advantages because the wild-type phenotype (possession of nitrate reductase and chlorate sensitivity) can be selected as ability to grow anaerobically on LN medium. It is proposed to adopt the terminology of *nar*$^+$ for wild-type (chlorate sensitivity) and *nar*$^-$ for all the mutants to be considered here. A consequence of this terminology is that *nir* can be reserved for the nitrite reductase marker.

Mapping with Mutants Selected as Nitrate Reductase-Less. The approximate position on the *E. coli* linkage map of several *nar*$^-$ mutants was determined by time of entry mapping with HfrH as donor and derivatives of AB1621 (e.g. T$^-$N98, *gal*$^-$*nar*$^-$*trp*$^-$) as recipients. In all cases the *nar* locus was transferred about 0.5 min after *gal* and 6 to 8 min before *trp*. A typical experiment is illustrated in Fig. 1. This order was further substantiated by cross-streak conjugation with F_2-*gal* as the donor. This episome transfers markers in the order *o* .... *gal* .... *suc*. None of these *nar* loci was transferred at high frequency by this F-prime particle

In further investigations *P1*-mediated transduction was used. Initially, *suc*$^+$, *gal*$^+$ and *bio*$^+$ transductants were selected using N6(HfrH) as donor and the polyauxotroph PA3476 as recipient (Table 1). The linkage between the donor *nar*$^-$ allele and the selected markers indicates that the *nar* locus is to the extreme right of this group. In addition, when the inheritance of non-selective donor markers (other than *suc*$^+$) by *gal*$^+$ transductants is considered, 25% gained *bio*$^+$ alone, 12% inherited both *bio*$^+$ and *nar*$^-$ and 4% inherited *nar*$^-$ alone. Assuming that the least frequent recombinant class arose from quadruple rather than double exchanges the gene order must be *gal* .... *bio* .... *nar*.

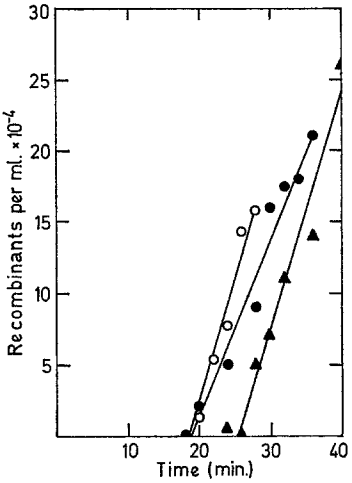

Fig. 1. Time of entry mapping of a *nar* locus. Streptomycin resistant recombinants which are *gal*$^+$ (○), *nar*$^+$ (●) and *trp*$^+$ (▲) were selected from a cross between HfrH (*gal*$^+$*nar*$^+$*trp*$^+$*str*^{s}, 2.5×10^8 per ml) and AB1621T$^-$N98 (*gal*$^-$*nar*$^-$*trp*$^-$*str*^{r}, 4×10^8 per ml)

Table 1. *Transduction mapping of a nitrate reductase locus*

P1 donor: HfrHN6 + + + *nar*$^-$

Recipient: PA3476 *suc*$^-$ *gal*$^-$ *bio*$^-$ +

Selected markers	Transductants per 10^6 *P1*	Number scored	Per cent transductants scored as			
			suc$^+$	*gal*$^+$	*bio*$^+$	*nar*$^-$
suc$^+$	5.3	116	—	48	16	9
gal$^+$	5.0	140	81	—	37	16
bio$^+$	6.6	104	19	27	—	56

A 3-factor reciprocal cross was next performed using *gal*$^+$ as the selective marker in each direction (Table 2). The number of *gal*$^+$*bio*$^+$*nar*$^+$ transductants relative to the total number of *gal*$^+$ transductants was much higher when the cross was performed in direction I (23%) than in direction II (1%). This indicates that the wild-type genotype arose by double exchange in direction I and by quadruple exchanges in direction II and this is only compatible with a gene order of *gal* .... *bio* .... *nar*.

Table 2. *Three-factor reciprocal cross at a nitrate reductase locus*

Direction I	*P1* donor:	HfrHN6	+	+	*nar*⁻
	Recipient:	W602	*gal*⁻	*bio*⁻	+
Direction II[a]	*P1* donor:	W602*gal*⁺	+	*bio*⁻	+
	Recipient:	W602*bio*⁺N6	*gal*⁻	+	*nar*⁻

Direction of cross	Selected marker	Transductants per 10^6 *P1*	Number scored	Per cent transductants scored as			
				bio⁺*nar*⁺	*bio*⁺*nar*⁻	*bio*⁻*nar*⁺	*bio*⁻*nar*⁻
I	*gal*⁺	29.7	124	23	23	50	4
II	*gal*⁺	13.0	83	1	67	17	15

[a] The participants in this cross were isolated as *P1*-sensitive transductants from a direction I cross.

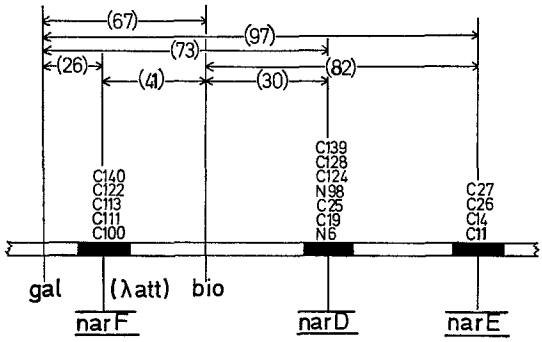

Fig. 2. Genetic map showing the relative positions of the three new *nar* genes. The map distances (100 minus per cent cotransduction) are averages for the mutant groups shown. Individual mutants comprising each group are represented as isolate numbers

The major significance of these results is that the mutants in question are not of the *narA* (*chlA*) type but belong to a new locus which we have designated *narD*.

Mapping of Mutants Selected as Chlorate-Resistant. To determine whether or not the isolation of *narD*⁻ mutants was conditioned by the new selection procedure a group of chlorate-resistant mutants were examined. The frequency of spontaneous mutation to chlorate resistance is at least 1 per 10^6 cells plated and this can be increased 100-fold by prior treatment with MNNG. By contrast, the reversion frequency of these *nar*⁻ mutants determined by plating on LN medium anaerobically is seldom higher than 1 per 5×10^7 cells plated. The majority of these mutants were isolated in W602 (*gal*⁻*bio*⁻) and these were used as recipients to W1485 (*gal*⁺*bio*⁺) in transduction crosses from which *gal*⁺, *bio*⁺ and *nar*⁺ recombinants were selected. From the linkage data obtained there appeared to be three different groups of mutant in the *gal* and *bio* region and these are shown in Fig. 2.

One of the groups gave map distances for *nar-gal* of between 65 and 84 (av. 73) and *nar-bio* distances of 24 to 44 (av. 30). In crosses between members of this group *nar*⁺ complete transductants were obtained at low frequencies but no abortive transductants could be detected although they were always found with W1485 as donor. Similar results were obtained when these mutants were crossed with the strains selected as *nar*⁻ by their failure to grow on LN-medium and

discussed above. It was therefore concluded that both selection procedures yield *narD*$^-$ mutants. Three factor crosses within the *narD* locus using *bio* as the outside marker indicated that the N6 site is further from *bio* than any of the other *narD*$^-$ mutants.

The second group of mutants was designated *narE*; it was characterized by a large *nar-gal* map distance (>92) coupled with a *nar-bio* distance of 70 to 90 (av. 82). Of four *narE* mutants, two (C11 and C14) gave a low frequency of complete transduction when crossed and the absence of abortive transductants indicated non-complementarity. So far the other mutants (C26 and C27) have not been tested for complementation and it is still possible that they could represent mutants at another closely-linked locus. The position of the *narE* gene was further defined using W1485 and HfrHN6 as donors and the 4 *narE* mutants as recipients; *nar*$^+$ was selective and the inheritance of *bio*$^+$, the non-selective marker of the donors, was scored. Typical results are presented in Table 3 and it can be seen that the presence of the *narD*-N6 mutation in the donor drastically reduces the linkage between the *narE*-C11 site and *bio*. This result would be expected only if *narD* lies between *bio* and *narE*.

Table 3. *Transduction mapping at the narE locus*

Recipient: W602C11 *gal*$^-$*bio*$^-$*narE*$^-$

P1 donor (*bio*$^+$)	Selected marker	Transductants per 10^6 *P1*	Number scored	Per cent transductants scored as *bio*$^+$
W1485 (*nar*$^+$)	*nar*$^+$	35	64	25
N6 (*narD*$^-$)	*nar*$^+$	15	61	5

Table 4. *Transduction at the narF locus*

P1 donor: W1485 + + +

Recipient: W602C122 *gal*$^-$ *narF*$^-$ *bio*$^-$

Selected marker	Transductants per 10^6 *P1*	Number scored	Per cent transductants scored as			
			gal$^+$*bio*$^+$	*gal*$^+$*bio*$^-$	*gal*$^-$*bio*$^+$	*gal*$^-$*bio*$^-$
nar$^+$	7	67	46	26	14	14
			gal$^+$*nar*$^+$	*gal*$^+$*nar*$^-$	*gal*$^-$*nar*$^+$	*gal*$^-$*nar*$^-$
bio$^+$	10	60	34	< 1.5	35	30

The third group designated *narF* was closest to *gal*, giving map distances ranging from 23 to 28 (av. 26) and *nar-bio* distances of between 38 and 48 (av. 41). Genetic homogeneity within this group was again demonstrated by the low frequency of complete *nar*$^+$ transduction and the absence of abortive transduction when crosses between constituent members were performed. The position of the *narF* gene was confirmed by analysing non-selective marker distribution in *nar*$^+$ and *bio*$^+$ transductants obtained with a wild-type donor and *narF* mutant recipients. Typical results (Table 4) show that amongst *bio*$^+$ transductants the

gal$^+$*narF*$^-$ genotype is least frequent indicating that it arose from quadruple rather than double exchanges. This is consistent only with the gene order *gal* .... *narF* .... *bio*, and non-selective marker distribution in the *nar*$^+$ transductants supports this conclusion.

Where performed, crosses between *nar* mutants from the 3 loci gave *nar*$^+$ transduction frequencies which conformed to the linkage map described (Fig. 2) and the ratios of abortive to complete transductants were as high or usually higher than those observed in crosses using wild-type donors.

Studies with Phage λ

In view of the capacity of λ for transducing *gal* and *bio* genes (MORSE, LEDERBERG and LEDERBERG, 1956; WOLLMAN, 1963) and the proximity of the *nar* genes to these loci, the possibility of specialized transduction of *nar* by λ was investigated. Initially, mutants of the *narD* class were examined and although they have received the greatest attention, the other classes of *nar* mutant were subsequently investigated.

Table 5. *Transduction of the nitrate reductase D locus by λ phage*

Experiment	Donor lysate (source and treatment)	Multiplicity of infection	*nar*$^+$ Transductants per plate with recipient		
			HfrHN6	W602C19	AT2687C25
I	None (broth control)	—	34	0	10
	W1485 (λ)	3.5	295	126	210
	W1485 (lytic growth)	3.5	0	0	0
	HfrHN6 (λ)	3.5	0	3	8
	AT2687C25 (λ)	0.8	1	0	0
II	None (broth control)	—	—	1	19
	W1485 (λ)	5.0	—	302	165
	W1485 (λ); 100,000 g supernate	3.5×10^{-3}	—	1	18
	W1485 (λ); 100,000 g pellet	3.2	—	170	103
	W1485 (λ); treated with λ antiserum	4.1×10^{-5}	—	2	15

Adsorption was in λ-broth and samples (0.1 ml) containing 2×10^8 recipient bacteria were plated in LN medium by the overlayer technique and incubated anaerobically for 3 days. Lysates were prepared by u. v. induction of lysogenic donor strains unless lytic growth is specified. In experiment IIa sample of the lysate was sedimented at 100,000 × *g* for 1 hr and the supernatant fluid and the pellet (resuspended in the original volume) were tested for plaque-forming and transducing activities. Another sample of the lysate was incubated with λ-antiserum for 45 min at 37° and assayed likewise.

Demonstration of narD Transduction by λ Phage. When lysate from an induced lysogenic *nar*$^+$ strain, W1485(λ) was incubated with three *narD*$^-$ mutants and selection for *nar*$^+$ made by plating on LN medium and incubating anaerobically, conversion to *nar*$^+$ occurred at a much greater frequency in the presence of λ than in its absence. Further experiments were performed to ensure that this was due to specialized transduction by λ. As shown in Table 5 (experiment I), "lytic λ" prepared by confluent lysis of a λ-sensitive *nar*$^+$ strain and induced lysate from lysogenic *narD*$^-$ strains were inactive. In addition, the plaque-forming and transducing activities of the transducing lysate were lost simultaneously by sedimenta-

tion or by treatment with λ-antiserum (Table 5, experiment II); both activities were recovered from the sedimented material. Thus it was concluded that transfer of the *narD* gene can be mediated by λ phage and at frequencies (1—4 transductants per 10^7 λ) comparable to the transduction of *gal* and *bio* by LFT-lysates. The relationship between the number of *nar*$^+$ transductants and amount of LFT-lysate plated is shown in Fig. 3 for AT2687C25, its λ-lysogen and W602C19. Over the proportional region the transduction frequency is 17.7 per 10^7 phage for

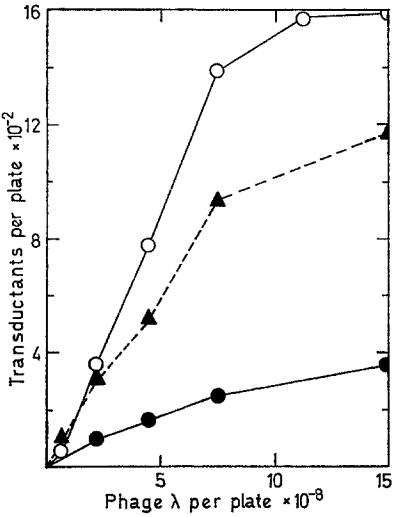

Fig. 3. Relationship between λ phage plated and *nar*$^+$ transductants produced. After adsorption samples containing 7.5×10^7 recipient organisms were plated; ○ AT2687C25; ● AT2687C25(λ) and ▲ W602C19

C25 but the frequency falls by a factor of 5 when the recipient is lysogenic. A saturation point is reached at 7.5×10^8 λ per plate (M.O.I. = 10), however, by doubling the amount of recipient, a higher saturation point is reached at 1.8×10^9 λ per plate with λ-sensitive C25 whereas little further increase in the yield of transductants is observed with the lysogen. Similar observations have been made for transduction of *gal* by LFT-lysates (MORSE *et al.*, 1956).

Linkage Analysis with narD Mutants. Transduction mapping with phage *P1* indicates that the *bio* marker is situated between *attλ* and the *narD* locus. Then according to the Campbell model (CAMPBELL, 1962), it would be predicted that when they are formed, the *narD*-transducing particles of an LFT-lysate should also possess the capacity for transducing *bio*. Consequently, a high frequency of cotransduction of *bio* with *narD* would be expected whereas, cotransduction of *gal* with *narD* would not be anticipated because these markers should be incorporated into opposite arms of the λ prophage. As predicted, cotransduction with *bio* was observed for 4 *narD*$^-$ mutants but never with *gal* (Table 6). Linkages between *narD* and *bio* of 21—56% were observed in different experiments with C25 as recipient, but they were always much lower with its lysogen (4.7—12.5%) and with C19 (<7.8%). When *bio*$^+$ was selective, this linkage varied between the same limits for C25, but was generally higher for C25(λ) and C19 (Table 6). In all cases the transductants were immune to λ when tested against *λgc*.

Table 6. *Cotransduction of narD with bio*

Recipients	Selective markers	Transductants per plate	Number tested	Per cent transductants scoring as			Heterogenotes carrying *narD*+
				bio+	*nar*+	*gal*+	
AT2687C25 (*gal*+)	*nar*+	603	437	42.4	—	—	3/319 (0.9%)
	bio+	741	147	—	30.6	—	4/116 (3.5%)
	nar+*bio*+	197	—	—	—	—	7/228 (3.1%)
AT2687C25(λ) (*gal*+)	*nar*+	428	277	11.2	—	—	2/341 (0.6%)
	bio+	145	64	—	25	—	5/64 (7.8%)
	nar+*bio*+	77	—	—	—	—	10/114 (8.8%)
W602C19 (*gal*−)	*nar*+	441	323	3.7	—	<0.3	
	bio+	86	64	—	10.9	<1.6	
	nar+*bio*+	16	—	—	—	—	
	gal+	2308	140	<0.7	<0.7	—	
W602C124 (*gal*−)	*nar*+	27	26	66	—	<3.9	
W602C128 (*gal*−)	*nar*+	34	35	45	—	<2.9	

Overnight cultures of recipient organisms were washed with 0.01 -M$MgSO_4$ by membrane filtration, resuspended at 3×10^9 bacteria/ml in the same fluid and incubated for 20 min at 37° with an equal volume of a phage suspension (1.5×10^{10}/ml) prepared by inducing W1485 (λ) and purifying the phage by sedimentation and dialysis versus λ-diluent. Transductants were selected by plating 0.1 ml samples as soft agar overlayers with the following media: *nar*+, LN medium (H_2/CO_2); *bio*+, LN-biotin (Air); *nar*+*bio*+, LN-biotin (H_2/CO_2) and *gal*+, galactose bromothymol blue nutrient agar. To reduce the faint background growth of the recipient on LN-biotin, avidin (Nutritional Biochemical Co.) was added to the soft top layers before plating (approximately 50 μg of protein after membrane filtering a suspension of avidin, 2.2 units/mg in λ-diluent).

The Occurrence of Transduction Heterogenotes for the narD Locus. An important feature of specialized transduction is the formation of heterogenotes which are transductants carrying an extra fragment of the bacterial genome in a defective or non-defective prophage. With phage λ some 70% of transductants for *gal* loci are heterogenotes (Morse, *et al.*, 1956), likewise with phage *Φ 80* 10—20% of *trp*+ transductants are heterogenotes (Matsushiro, 1963). The heterogenotes are characterized by their genetic instability and the ability to yield high frequency transducing (HFT) lysates upon induction. In the case of *narD* and λ, heterogenotes were not discovered until a method had been devised for screening large numbers of *nar*+ transductants (see Materials and Methods).

Transductants of AT2687C25 and its λ-lysogen have been studied in greatest detail and less than 1% of *narD*+ transductants were detectably heterogenotic (Table 6). All 5 heterogenotes selected primarily as *nar*+ were also *bio*+, and they are presumed to have the genetic constitution: *bio*−, *narD*−/(λ-*bio*-*narD*)+. Significantly, the proportion of heterogenotes was increased when *nar*+*bio*+ cotransductants were selected directly (Table 6) and compared with the *nar*+ selection, this increase is directly related to the enrichment of *nar*+*bio*+ transductants. The incidence of these heterogenotes was also higher when *bio*+ was alone selective (Table 6), and in terms of the number of *nar*+*bio*+ in these samples the proportion of heterogenotes is as high as 11.3% for C25 and 31.0% for C25(λ). Since the same

species of exogenote are being considered whether *bio*$^+$ or *nar*$^+$ are selective and since the similar selective media are used for both, this significant increase in heterogenote yield may be related to the aerobic selection of *bio*$^+$ compared to the anaerobic selection of *nar*$^+$.

Assuming that *bio* lies between *narD* and *attλ*, a further prediction based on the Campbell model is that all heterogenotes selected as *nar*$^+$ possess an exogenote of the type *λ-bio-narD* whereas the converse would not be expected i.e. heterogenotes selected as *bio*$^+$ need not necessarily contain *narD* in the exogenote but may be either *λ-bio* or *λ-bio-narD*. (This also assumes that recombination between *λ* and transducing *λ* is rare.) As mentioned above, all the *nar*$^+$-selected heterogenotes were *bio*$^+$, but of the *bio*$^+$-selected transductants, 9 were heterogenotic with respect to *narD* (Table 6), and when screened for the presence of heterogenotes on the basis of high frequency transfer of *bio*$^+$, 2 more heterogenotes were detected, in addition to the 9 *bio*$^+$*narD*$^+$ heterogenotes, and these were *bio*$^+$*nar*$^-$. Clearly, exogenotes of the *λ-bio* and *λ-bio-narD* types exist and, despite the smallness of the sample, the absence of the *λ-narD* type is consistent with the theory of transducing phage origin based on the Campbell model. Alternately, if the Campbell model is assumed, the results confirm the gene order *gal-λ-bio-narD*.

Properties of Heterogenotes. All the *narD*$^+$ heterogenotes examined proved to be unstable; they segregate haploid *narD*$^-$ derivatives and some haploid *narD*$^+$ recombinants. The heterogenotes were sensitive to chlorate anaerobically and the *narD*$^-$ segregants were not only unable to grow on LN medium but also chlorate resistant, indicating that the wild-type allele *narD*$^+$ and chlorate sensitivity is dominant over *narD*$^-$ and chlorate resistance.

A total of 14 heterogenotic strains derived from AT2687C25 and its *λ*-lysogen were examined for HFT-lysate formation after u.v.-induction. The lysates contained 1.5—5.6 $\times 10^9$ plaque-forming units per ml whereas the transducing phage titres assayed in the presence of helper phage ("lytic *λ*") were lower by factors from 65 to 2,300: representative results are shown in Table 7. The conditions chosen for assaying transducing *λ* were those which gave the highest values; lower titres were obtained with a lysogenic recipient alone, and with non-lysogenic AT2687C25 in the absence of helper the HFT-lysates were virtually inactive at

Table 7. *Plaque-forming and transducing activities of HFT-lysates*

Hetero-genote	Parent	Selection	Transducing activity (T) (phage per ml $\times 10^{-7}$)	Plaque-forming activity (P) (phage per ml $\times 10^{-9}$)	Ratio T/P
HN2	AT2687C25	*nar*$^+$	1.27	2.84	1/224
HNB5	AT2687C25	*nar*$^+$*bio*$^+$	3.62	2.36	1/65
HNB11	AT2687C25	*bio*$^+$	0.84	2.70	1/321
HN15	AT2687C25(*λ*)	*nar*$^+$	0.19	3.41	1/1790
HNB20	AT2687C25(*λ*)	*nar*$^+$*bio*$^+$	3.74	3.66	1/98
HB29	AT2687C25(*λ*)	*bio*$^+$	0.99	1.98	1/200

Single colonies of heterogenotic strains on *λ*-agar plates which gave a positive heterogenote test were induced with u. v. radiation and the resulting lysates were assayed for plaque-forming activity and for transducing activity with added helper phage.

the highest dilutions (Fig. 4). The absolute titres for the transducing particles may well be higher because the transduction efficiency under the test conditions is probably less than 1 (ARBER, 1958). Since similar results were obtained with all the HFT-lysates, the requirement for helper phage indicates that all the transducing phage examined are defective and can be designated *λdnD*. The production of HFT-lysates from heterogenotes derived from non-lysogenic C25 was not dependent upon superinfection with λ so all the heterogenotes examined can be regarded as double lysogens (*λ,λdnD*).

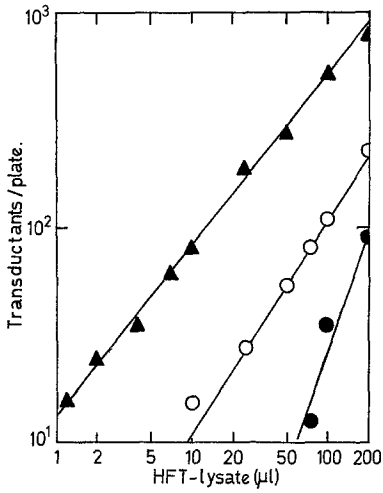

Fig. 4. The transducing activity of HFT-lysate when assayed with: *narD*⁻, •; *narD*⁻(λ⁺), ○; *narD*⁻ with λ⁺ as helper, ▲. The lysate from a heterogenotic *narD*⁺ transductant of AT2687C25, HN1 contained 1.8×10^9 plaque-forming units per ml and 5.6×10^6 transducing units per ml; dilutions were prepared so that 0.1 ml samples contained the volume of lysate shown

Using HFT-lysates the incidence of heterogenotes amongst *narD*⁺ transductants obtained from a lysogenic recipient and from non-lysogenic recipient plus helper was no greater than with LFT-lysates. So far, no heterogenotic transductants have been obtained with HFT-lysate and non-lysogenic recipient alone.

The presence of two species of phage particle in the HFT-lysates was confirmed by CsCl density-gradient centrifugation. Four lysates were examined and in each case a band of transducing activity was found displaced or separated from the plaque-forming material. The lysates from heterogenotes HN2 and HN15 contained transducing particles (*λdnD*) which had densities 0.0014 and 0.0028 greater than λ respectively. On the other hand, densities 0.0180 and 0.0054 less than λ were found for the *λdnD* particles in HN1 and HNB5 HFT-lysates respectively; representative profiles are shown in Fig. 5.

Transduction Studies with other nar⁻ Mutants. This investigation of *nar* transduction by LFT-lysates of λ was extended to include a total of 70 *nar*⁻ isolates. With 12, a relatively high reversion frequency obscured the results but of the remainder 20 were transduced to *nar*⁺ and 38 were not. The latter group included the 4 mutants designated earlier as *narE* mutants, and some which are

neither *narD*, *E* nor *F* mutants. Only 11 of the transducible strains have been characterized and they comprise 6 isolates already designated as *narD* mutants and the 5 *narF* mutants. Transduction of the *narF* gene by λ is consistent with its location between *gal* and *bio*. With mutants C113 and C122, joint transduction of *nar* with *gal* but not *bio* (Table 8) indicates that the corresponding *narF* loci are situated between *gal* and *att*λ. The situation with regard to the other *narF* mutants, C100 and C111, is more complex because here linked transduction of

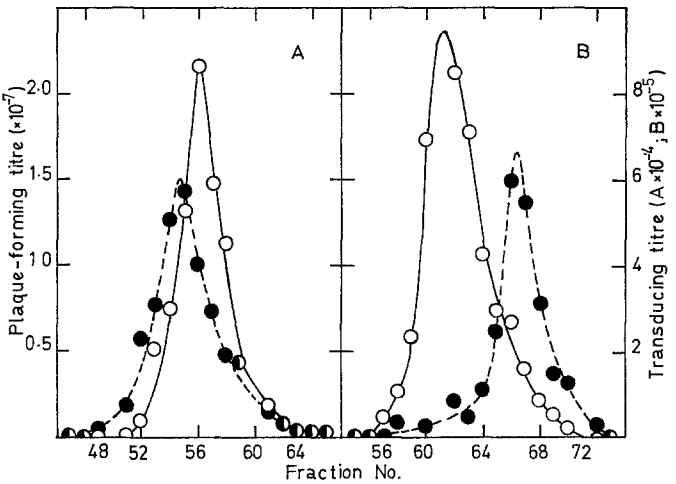

Fig. 5. Density gradient centrifugation of HFT-lysates. Plaque-forming (○) and transducing (●) activities are shown for lysates from heterogenotes A, HN2 and B, HNB5

Table 8. *Transduction of narF by phage* λ

Recipients (*gal*⁻, *bio*⁻, *narF*⁻)	Transductants (*nar*⁺) per plate	Number tested	Per cent transductants scoring as	
			gal⁺	*bio*⁺[a]
W602C100	72	139	38	8
W602C111	66	126	83	83
W602C113	302	100	14	<1
W602C122	480	100	25	<1

[a] All the *nar*⁺ transductants which inherited *bio*⁺ as a non-selective marker were also *gal*⁺.

The donor lysate prepared from W1485(λ) was mixed with an equal volume of overnight culture of recipient organisms (M.O.I. = 5); *nar*⁺ transductants were selected by plating 3×10^8 bacteria by the overlayer technique.

nar with *gal* and *bio* was observed (Table 8). Compared with mutant C122 in the same group, these mutants behaved differently in conjugation with F'_2-*gal* using the cross-streak test; C122 gave *nar*⁺ recombinants but C100 and C111 did not. Presumably, the C122 site lies close to *gal* and the corresponding segment of chromosome is contained on the episome whereas the C100 and C111 mutations are either further from gal or they extend beyond the region transferred by the episome.

Discussion

The nitrate reductaseless mutants examined here fall into three distinct groups (*narD*, *E* and *F*) based on linkage analyses and complementation tests (abortive transduction) with phage *P1*. Like one of the previously described nitrate reductase loci, *chlA* (*narA*) these groups are situated in the *gal* and *bio* region of the *E. coli* linkage map. The *narA* gene is thought to lie between *gal* and *bio* and more specifically between *attλ* and *bio* because *gal* and *narA* are not cotransduced by LFT-lysates of *λ* (PUIG *et al.*, 1967). This clearly distinguishes *narA* from the three *gal* and *bio*-linked genes considered here, because they either map outside the *gal-bio* segment (*narD* and *E*) or are cotransducible with *gal* (*narF*). When a large sample of mutants has been examined we may find some of the *narA* type and more also of the *narB* and *narC* types. Mutants selected as nitrate reductaseless (N) are not genetically distinct from those selected as chlorate resistant (C) because both methods yield non-complementary *narD* mutants. So far all the nitrate reductase selected mutants have been *narD*$^-$ but again, when more have been examined in detail we expect to find representatives from the other loci. The relatively high frequency of *nar*$^-$ mutation may be the cumulative effect of normal frequencies in many genes, in which case further *nar* genes may await discovery. Of the activities affected in the *nar*$^-$ mutants, nitrate reductase and formate: ferricytochrome b_1 oxido-reductase are membrane associated (VENABLES *et al.*, 1967, 1968) and the existence of a multiplicity of genes affecting these functions is not necessarily surprising.

Transduction of the *narD* and *narF* loci by phage *λ* is consistent with its function as a specialized transducing phage, restricted to transferring genes situated close to the prophage attachment site, Except for a few minor details the characteristics of the *narD* transducing system are essentially similar to those of the *gal*, *bio* (*λ*) and *trp* (Ø80) systems. The most significant difference would seem to be the relatively low frequency of heterogenote formation both with LFT and HFT lysates. This may be a reflection of the particular deletions of *λ*-genome required to accommodate the *attλ-narD* region or it may be a feature of the *nar*$^+$ selection procedure. The relative orientation of *narD* and *λ* prophage is similar to that for *bio* and *λ* or *trp* and *Ø80*, where both defective and non-defective transducing particles have been found. Here 14 independently-isolated heterogenotes contained defective transducing phage. However, insertion of the *attλ-narD* region into transducing particles necessitates considerably greater deletions of *λ*-DNA (and therefore a greater possibility of deleting essential *λ* functions) than for the insertion of *bio*. As yet, the transduction of *narF* by *λ* has not been studied in detail; the cotransducibility of *gal* with *narF* indicates that *narF* is to the left of *attλ*. However, the cotransducibility of *gal*, *narF* and *bio* with 2 mutants, C100 and C111, demands further investigation. Similar linked transduction of *gal* with *bio* has been described by FUERST (1966) with a recipient deleted for *attλ*. It is conceivable that C100 and C111 (both spontaneous mutants) have deletions in the *narF* gene which extend through the *attλ* region. The conjugation studies with F'_2-*gal* lend a little support to this possibility. Despite being linked to *bio* and *gal* by *P1* transduction the *narE* gene appears to be too far from *attλ* either to be incorporated into a transducing particle or, for the generation of such a particle to occur at a high enough frequency for it to be detected.

During the course of this work, transduction of nitrate reductase sites by 2 defective biotin transducing phage (*λdb*) has been reported (ADHYA, CLEARY and CAMPBELL, 1968). This finding together with additional genetic evidence presented by these authors suggests that their mutants are of the *narD* type.

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Dr. JOHN R. GUEST
Department of Microbiology Sheffield University Western Bank
Sheffield, S 10 2 TN/England