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Biochemical and Genetic Studies with Nitrate Reductase *C*-gene Mutants of *Escherichia coli*

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Summary. Twenty-eight *narC* (*chlC*) mutants of *Escherichia coli* were isolated and characterised by their resistance to chlorate, inability to use nitrate as terminal electron acceptor and positive gas reaction. The extent of gas production by the majority of mutants was almost normal but quantitative differences ranging from 40 to 100% of wild-type activity were found. Biochemical studies showed that all the mutants lacked nitrate reductase, decreasing gas production was correlated with a simultaneous decrease in formate dehydrogenase activity and the lowest gas production was due to deficiencies in formate dehydrogenase and hydrogenase. The position of *narC* relative to other loci was determined as: *purB* .... *hemA* .... *narC* .... *supIII,C* .... *galU* .... *attΦ80* .... *tonB* .... *trp* .... *cysB* by transduction analysis, and the mutant sites of 6 strains representing the complete range of gas reactions were clustered at this position. It is suggested that *narC* is the structural gene for nitrate reductase and the variations in phenotype may be due to polarity effects on neighbouring genes specifying components of the formate hydrogenlyase system. Transduction of *narC* by *Φ80* could not be detected but an effect of *galU*$^-$ on phage *P1kc* susceptibility was demonstrated.

Introduction

The reduction of nitrate by *Escherichia coli* is catalysed by a membrane-bound enzyme, nitrate reductase, which is associated with cytochrome b_1 and formate dehydrogenase (Iida and Taniguchi, 1959; Itagaki, Fujita and Sato, 1962; Azoulay, Puig and Pichinoty, 1967; Showe and DeMoss, 1968). The enzyme is a component of an anaerobic electron transport system with nitrate as terminal acceptor and it is induced by nitrate and repressed by oxygen. This enzyme system also reduces chlorate to chlorite with lethal consequences. Mutants lacking nitrate reductase have thus been isolated by virtue of their resistance to chlorate (Piechaud, Puig, Pichinoty, Azoulay and Le Minor, 1967) and by their failure to use nitrate as terminal electron acceptor (Venables and Guest, 1968) during anaerobic growth. Studies with these mutants indicate that mutations in at least five genes affect nitrate reductase activity and the corresponding loci *nar* or *chl* have been mapped as shown in Fig. 1. Some confusion over the alphabetical nomenclature for these genes has arisen because the *A* gene was originally placed between *attλ* and *bio* (Puig, Azoulay and Pichinoty, 1967) and before this was corrected (Puig, Azoulay, Gendre and Richard, 1969) other letters, *D*, *E* and *F*, were assigned to the three loci in the *gal* region (Venables and Guest, 1968; Adhya, Cleary and Campbell, 1968). It is now proposed to adopt the assignments shown in Fig. 1 and in consequence the *A* and *D* genes correspond respectively to the *λ*-transducible *narD* and *narF* loci of Venables and Guest (1968); the *narE* designation remains unchanged. It is also proposed to retain *nar*$^-$ for describing nitrate reductase mutants but *nar*$^-$ and *chl*$^-$ (chlorate resistance) may be regarded as synonymous.

Interest in nitrate reductase stems from its multicomponent particulate nature and recent studies have shown that an active and sedimentable complex can be reconstituted from soluble components of *A* and *B* mutants (Azoulay, Puig and Couchoud-Beaumont, 1969) or *A* and *E* mutants (W. A. Venables, personal communication). Mutants with defects in four of the genes, *narA*, *B*, *D* and *E*, exhibit a pleiotropic phenotype and in particular, they lack formate hydrogenylase as well as some form of nitrate reductase activity. This is readily apparent from their failure to produce gas during anaerobic growth. Such effects may stem from defects in enzymatic and structural components or assembly factors common to both enzyme systems.

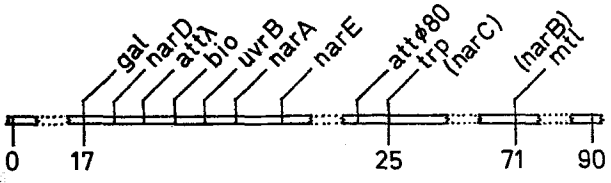

Fig. 1. Linkage map of *Escherichia coli* showing the nitrate reductase loci (*nar=chl*); based on studies of Puig, Azoulay and Pichinoty (1967), Venables and Guest (1968), and Adhya, Cleary and Campbell (1968). Map distances are shown in min according to Taylor and Trotter (1967)

By contrast, the *narC* mutant, originally described by Puig and Azoulay (1967) and located near the *trp* operon, lacks nitrate reductase but possesses formate hydrogenlyase. Consequently, the *narC* gene was thought to be the structural gene for the nitrate reductase component of the enzyme complex. In order to investigate this possibility further, the present work describes biochemical studies with a group of *narC* mutants which differ in their capacities for gas production. Also, an examination of the linkage map of *E. coli* (Taylor and Trotter, 1967) reveals certain relationships between genes mapping near the attachment sites for the lamboid phages λ and $\Phi 80$; genes concerned with galactose metabolism, suppressor loci, as well as *nar* (*chl*) loci are found in both regions. Hence, transduction studies, using phages *P1* and $\Phi 80$ and the same group of *narC* mutants, designed to locate the *narC* gene more precisely and to investigate whether it is transduced by $\Phi 80$ are also reported.

Methods

Bacteria and Phages. Mutants were selected in the following suppressor-free strains of *Escherichia coli* K12: W3110, prototroph(W), W3110 *gal*$^-$, *trpA*$^-_{amber}$(WGA) and W3110 *gal*$^-$, *trpA*$^-_{amber}$, *cysB*$^-$(WGAC) which were obtained from Dr. W. J. Brammar. Other strains containing markers used in the genetic studies were: M226 (*F*$^-$, *galU*$^-$, *str*^{r}), CA275 (*HfrC*, *lac*$^-$, *trp*$^-_{amb}$, *supIII*$^+$), CA299 (*HfrC*, *lac*$^-$, *trp*$^-_{amb}$, $\Phi 80$ $\Phi 80$ *dsupIII*$^+$*galU*$^+$); X252 (*F*$^-$, *lacY*$^-$, *galU*, *str*^{r}, $\Phi 80$ $\Phi 80$*dgalU*$^+$) and X339 (*F*$^-$*ilv*$^-_{ochre}$, *his*$^-_{amb}$, *arg*$^-$, *galU*$^-$, $\Phi 80$ $\Phi 80$ *dsupC*$^+$*galU*$^+$) were from Dr. S. Brenner. W3110 *tonB*$^-$, *trp*$^-_{del}$, *supC*$^+$ was from Dr. W. J. Brammar. S729 (*F*$^-$, *met*$^-$, *hemA*$^-$) and S730 (*F*$^-$, *thi*$^-$, *trp*$^-$, *his*$^-$, *tyr*$^-$, *pyr*$^-$, *purB*$^-$, *hemA*$^-$, *gal*$^-$, *lac*$^-$, *str*^{r}) were obtained from Professor A. H. Stouthamer and 0254 (*F*$^-$, *thi*$^-$, *trp*$^-$, *his*$^-$, *tyr*$^-$, *purB*$^-$, *lac*$^-$, *gal*$^-$, *xyl*$^-$, *mal*$^-$, *tonA*$^-$, *tsx*$^-$, *str*^{r}) was from Professor P. G. deHaan. *Gal*$^+$ and *gal*$^+$, *trp*$^+$ derivatives of 0254 and S730 were prepared by conjugation using HfrH as donor and selection on appropriate minimal media containing streptomycin. Phages *P1kc*, $\Phi 80$ and $\Phi 80h$ were obtained from Dr. C. Yanofsky and *P1vir1* was from Dr. J. Tomizawa.

Media. The minimal media were: Medium E of Vogel and Bonner (1956) with glucose (0.2%) as carbon source or galactose (0.5%, plus bromothymol blue) for *gal*$^+$ selection, enriched with Bacto Nutrient Broth (0.2% v/v) in transduction studies and, Medium LN, the lactate-nitrate medium of Venables and Guest (1968). Both media were supplemented with vitamins and amino acids according to the requirements of the strain to be grown or selected.

Bacterial stocks were grown and maintained in L-broth and L-agar (Lennox, 1955), supplemented with cysteine (50 μg/ml) or δ-aminolevulinate (40 μg/ml, sterilized by filtration) for *cysB*$^-$ and *hemA*$^-$ mutants respectively.

Selection of Mutants. Mutant derivatives of strains W, WGA and WGAC were selected by their resistance to chlorate. Cultures, with or without prior treatment with *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine, were plated on chlorate medium (Venables and Guest, 1968) and incubated anaerobically at 37° for 20 hr. Colonies of resistant organisms were then purified on glucose minimal medium and single colonies tested again for their resistance to chlorate, their ability to grow anaerobically on LN medium and ability to produce gas. Gas-producing *narC* mutants of independent origin were numbered with a C prefix.

Test for Gas Production. Tubes (16×125 mm) containing 12 ml of L-broth plus extra glucose (1% w/v) and Durham tubes (6×30 mm) were inoculated with 0.1 ml of an 18 hr culture of the test organism and incubated without shaking for 40 hr at 37°. A positive result is indicated by the presence of a gas bubble in the Durham tube and the method was quantitated by measuring the height of the bubble with a travelling microscope. The averages of quadruple determinations are recorded in mm.

Enzymatic Analyses. Organisms were grown for 18 hr at 37° from 0.5% inocula in stationary Erlenmeyer flasks $^3/_4$-filled with medium (peptone 0.4%, yeast extract 0.4%, K_2HPO_4 0.6%, glucose 1% added after autoclaving, final pH 6.8). Cultures were harvested and washed twice in cold potassium phosphate buffer (0.01 M, pH 7.4) by centrifuging. Aqueous suspensions (6 mg dry wt./ml) were used for experiments with whole organisms or buffered suspensions (80—100 mg dry wt./ml) were stored at −14° for disruption in a Hughes press. Crude extracts, cell wall-membrane and soluble fractions were prepared according to Gray, Wimpenny, Hughes and Mossman (1966). Protein was measured as described by Lowry, Rosebrough, Farr and Randall (1951) with bovine serum albumin as standard.

Formate hydrogenlyase, formate dehydrogenase (MB, with methylene blue and BV, with benzylviologen as electron acceptor) and hydrogenase were assayed manometrically at 37° with whole organisms using the methods quoted by Gray *et al.* (1966); activities are expressed as μl gas evolved or absorbed/mg dry wt. organisms/hr.

Formate:nitrate oxidoreductase was assayed in crude cell extracts by measuring formate-dependent reduction of nitrate to nitrite. Extracts were incubated at 25° in 0.1 M potassium phosphate buffer (pH 7.4) with formate (2 mM) and nitrate (2 mM), and samples were withdrawn at 5 min intervals for colorimetric determination of nitrite. Results are expressed as μmoles nitrite formed/mg protein/hr.

The NADH:nitrate and reduced benzylviologen (BVH):nitrate oxidoreductases of crude extracts were assayed spectrophotometrically at 25° under anaerobic conditions (Cole and Wimpenny, 1968; Wimpenny and Cole, 1967). The corresponding NADH:chlorate and BVH:chlorate oxidoreductases were assayed similarly except that nitrate was replaced by chlorate. Activities are expressed as μmoles NADH oxidised/mg protein/hr and △O.D. at 580 mμ (BVH oxidised)/mg protein/hr.

The cytochrome b_1 contents of the cell wall-membrane fractions and the cytochrome c_{552} of the soluble fractions were measured by difference spectra (dithionite reduced *vs.* ferricyanide oxidised; b_1, 559—575 mμ, and c, 552—536 mμ) with an SP800 spectrophotometer (Gray *et al.*, 1966). The results are expressed as mμmoles cytochrome per mg protein.

Transduction with Phage P1. The media and methods of Lennox (1955) were employed. Lysates of donor strains were prepared by confluent lysis. Transduction mixtures contained per ml: 10^9 stationary phase recipient organisms, 2×10^9 *P1kc* or 5×10^7 *P1vir1* in L-broth and $CaCl_2$ (5 μmoles). The bacteria were preincubated with the $CaCl_2$ for 30 min at room temperature followed by a 30 min incubation at 37° with phage. Unadsorbed phage was removed by centrifuging and appropriate dilutions of the transduced culture were plated on selective media.

Transduction with Phage Φ80. Lysates of *Φ80* were prepared by inducing log phase cultures of W3110(*Φ80*) or other lysogenic donor strains. For transduction, log phase cultures of recipient bacteria (10^9/ml) growing in λ broth (Venables and Guest, 1968) were resuspended in the same volume of 0.01 M-$MgSO_4$ and aerated for 30 min at 37°. Phage was added at a multiplicity of 6 and after adsorption for 20 min at 37° without aeration, mixtures were plated on appropriate selective media.

Linkage Analysis. For scoring the inheritance of non-selective markers, transductant colonies were patched on the corresponding selective medium and replicated on to appropriate test media. Chlorate and LN media were both used for scoring *nar* markers and the most suitable medium for selecting and scoring *hemA*$^+$ was nutrient agar (Oxoid Nutrient Broth No. 2 solidified with 1.5% agar).

Results

Properties of narC Mutants

Isolation and Characterisation. Because of the reported linkage of *narC* with *trp*, mutants were isolated in *trp*$^-$ and *cysB*$^-$ parents (WGA, WGAC) as well as in the prototroph (W). Selection was primarily for resistance to chlorate and *narC* mutants were distinguished from other *nar*$^-$ strains by their ability to produce gas (*narA*, *B* and *E* mutants are anaerogenous though some *narD* mutants did give an extremely poor gas reaction). It was found that *narC* mutants represented only 0.5—2% of spontaneous chlorate resistant isolates but the proportion increased to approximately 20% following treatment with mutagen. Some 28 independent mutants (C31—C58) were isolated and like other *nar*$^-$ strains they failed to grow or grew very poorly on LN medium (under H_2 with 5% CO_2). They all grew on glucose minimal medium under aerobic and anaerobic conditions. A striking feature of these mutants is the variation in gas production which ranges from 40—100% of wild-type. The majority (20) had more than 80% of wild-type activity, 6 were in the 60—80% range and 2 gave between 40—60% of wild-type gas production in the standard test. The capacity for gas production appeared to be a stable and reproducible characteristic of each *narC* mutant. Revertants selected on LN medium were obtained at normal frequencies and wild-type gas production and chlorate sensitivity were restored simultaneously. Also, transfer of *narC* mutations into other backgrounds by transduction resulted in simultaneous transfer of inability to grow on LN medium, chlorate resistance and the corresponding gas reaction. These observations favour the view that the mutants are produced by single mutational events (which may be leaky or have pleiotropic or polar effects); however, the possible existence of closely-linked multiple mutations has not been ruled out.

Some of the mutants differed in other respects, e.g. some mutants grew poorly on chlorate medium, others gave a poor but significant response on LN medium and one mutant was sensitive to EDTA (5 mM). However, no systematic patterns of behaviour could be established. In further tests for altered surface properties all the mutants exhibited wild-type resistance to deoxycholate (1%) and methylene blue (0.01%).

Biochemical Studies. Selected mutants representing the overall spectrum of gas reactions were chosen for detailed biochemical analysis (Table 1). All the mutants lacked nitrate reductase activity when assayed with formate, NADH or reduced benzylviologen (BVH) as reductants. They were correspondingly deficient in chlorate reductase and this was most apparent with BVH as electron donor.

Table 1. *Biochemical analysis of narC mutants*[a]

	Strain						
	W3110	W-C55	WGA-C31	WGA-C57	W-C39	W-C54	W-C38
Gas production	11.2	10.9	10.5	9.9	8.4	7.8	4.7
Formate hydrogenlyase	7.3	7.5	7.8	6.4	6.1	2.4	0.2
Formate dehydrogenase (BV)	9.5	7.9	10.3	9.3	8.5	1.9	0.4
Formate dehydrogenase (MB)	15.6	12.7	16.5	13.5	7.8	5.4	1.9
Hydrogenase	79	72	83	67	78	70	1.5
Formate:nitrate oxidoreductase	0.62	0	0.04	0.01	0	0	0
BVH:nitrate oxidoreductase	128	0.8	0.9	2.2	2.0	1.2	0.2
NADH:nitrate oxidoreductase	0.90	0	0.02	0	0.09	0	0
BVH:chlorate oxidoreductase	583	78	38	59	38	39	30
NADH:chlorate oxidoreductase	0.92	0.13	0.13	0.18	0.23	0.02	0.23
Cytochrome b_1	0.27	0.17	0.20	0.21	0.26	0.11	0.12
Cytochrome c_{552}	0.013	0.008	0.015	0.012	0.011	0.008	0.014

[a] The assay methods and the units employed are described in the Methods section.

Also, two of the mutants noted for their partial sensitivity to chlorate (W-C55 and WGA-C57) had more than 10% of wild-type BVH:chlorate oxidoreductase activity. The decreasing capacity for gas production was paralleled by decreasing specific activities for formate hydrogenylase and of its component reactions (formate dehydrogenase and hydrogenase) this trend was correlated most closely with the formate dehydrogenase activities (Table 1). Hydrogenase appeared to be unaffected except in W-C38 which was deficient in both dehydrogenase and hydrogenase activities. However, despite the relatively low formate hydrogenlyase activity of this mutant it was capable of accumulating a considerable amount of gas under the conditions of the gas test. Variations in the cytochrome b_1 contents of the cell wall-membrane fractions of the mutants were observed (Table 1). However, a quantitative assessment of these values is difficult because of the nature of the procedure for isolating the sub-cellular fractions. Nevertheless, some of the mutants appear to possess less cytochrome b_1 than wild type and this was confirmed by measurements with whole cells. No significant changes in the soluble cytochrome c_{552} were associated with the mutations.

These results indicate that the primary effect of the *narC* mutations is in the terminal stages of electron transport to nitrate (BVH:nitrate oxidoreductase) and that changes in formate dehydrogenase, hydrogenase and possibly cytochrome b_1 may be secondary consequences as are the effects on formate:nitrate and NADH:nitrate oxidoreductase activities.

Transduction with Phage P1

Of the 28 *narC* mutants selected for study, 23 were transduced to *nar*$^+$ with phage *P1kc* at normal frequencies by selection on LN medium. The 5 remaining mutants were not transduced at frequencies greater than 3% of normal, and they have not been investigated further. In all cases of *narC* transduction, linkage

with a *trp* marker was demonstrated and the presence of abortive *nar*$^+$ transductants was observed. The results of genetic studies designed to determine the precise location of *narC* are summarised in Fig. 2. Nearly all the crosses have been performed with both the C31 and C38 mutations, which represent the high and low limits of gas production found amongst *narC* mutants. However, no significant differences were observed and details are given for representative crosses only (see below). Likewise, many crosses involving other *narC* mutants have been performed and although they are not reported in detail, the results are included in the average map distances shown in Fig. 2.

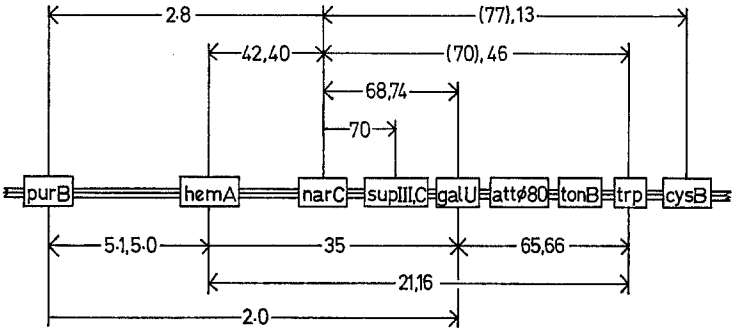

Fig. 2. Genetic map of the *purB* to *cysB* region. The linkages, which are inversely related to map distance, are the averages for cotransduction frequencies (%) obtained in all experiments. Where transduction crosses were performed in both directions the head of each arrow points to the selective marker (wild-type allele in the donor) and the corresponding linkage is placed nearest to it. The values in parentheses are considered unreliable due to interference from the non-selective marker

Linkage Analysis. In preliminary experiments linkages between *narC* and *trpA* were determined and some difficulty was experienced when selecting *nar*$^+$ on supplemented LN medium when *trp*$^-$ was non-selective. This is apparent from the discrepancies between the linkages obtained in the same experiment with *nar*$^+$ compared with *trp*$^+$ as selective marker (Table 2). Cotransduction of *trp* with all the *narC* mutant loci was invariably between 40 and 52% with *trp*$^+$ selective and between 55 and 95% with *nar*$^+$ as selective marker, and the former values were considered the more reliable. It appears that some selection for the wild-type allele of the amino acid marker occurs during selection of *narC*$^+$ on LN medium. Similar difficulties were experienced with *cysB* as non-selective marker (Table 2) but not with *hemA* or with *bio* (used previously with other *nar* mutants). It is not known whether this effect is specific for *narC* mutants or whether impaired utilisation of supplements is a general feature of growth on the LN medium.

Since the linkage of *narC* to *trpA* was greater than its linkage to *cysB*, *narC* and *cysB* were placed on opposite sides of the *trp* locus, and this order was confirmed by examining the relationships between *narC* and the *tonB* and *attΦ80* loci as well as by three factor crosses and transduction with *Φ80* (see below). Three different T_1-resistant mutants with deletions extending from *tonB* into the *trp* operon were all chlorate-sensitive and capable of growth on LN medium indicating that *narC* is not between *tonB* and *trp*. In further studies the effect

Table 2. *Linkage relationships between narC and neighbouring loci*

Donor (*P1kc*)	Recipient	Selective marker	Transductants per 10^6 phage	Number scored	Inheritance of non-selective donor markers	
					Marker	Linkage (%)
W	WGA-C31	*trpA*+	41	128	*narC*+	42
W	WGA-C31	*narC*+	24	100	*trpA*+	80
W	WGAC-C43	*cysB*+	66	179	*narC*+	14
W	WGAC-C43	*narC*+	12	106	*cysB*+	77
W	WGA-C31 (*Φ80*)	*trpA*+	5.4	100	*narC*+	40
W (*Φ80h*)	WGA-C31 (*Φ80*)	*trpA*+	3.0	100	*narC*+	1
W *trp*$^-_{del}$ *supC*+	WGA-C31 (*Φ80*)	*supC*+ (*trp*+)	5.3	100	*narC*+	72
CA275 *trp*$^-$ *supIII*+	WGA-C31 (*Φ80*)	*supIII*+ (*trp*+)	9.7	111	*narC*+	68
W-C38	M226	*galU*+	10	120	*narC*−	77
W	M226 *trp*+C38[a]	*narC*+	8.6	108	*galU*+	63
W-C38	S729	*hemA*+	9.5	96	*narC*−	45
S729	W-C31[b]	*narC*+	29	120	*hemA*−	41
W-C38	0254 *gal*+	*purB*+	2.9	156	*narC*−	3.2

[a] This *gal*−*narC*− recipient was found amongst *trp*+ transductants selected from the W-C38 × M226 cross.

[b] W-C31 is a *P1*-sensitive derivative of WGA-C31 obtained by selecting for *gal*+ and *trpA*+ recombinants in consecutive *P1vir1* transductions with strain W as donor.

of the presence of a *Φ80* prophage on the *narC* to *trpA* linkage was investigated (Table 2). Using a *Φ80* lysogen as recipient the *narC* to *trpA* linkage fell from 40% to approximately 1% when the donor strain was lysogenic for *Φ80h*. This established that *narC* and *trpA* are on opposite sides of *attΦ80*. Similar demonstrations of lysogeny increasing the apparent distance between pairs of markers spanning a prophage have been made by Rothman (1965) and Signer (1966). As a consequence of these observations the relationships between *narC* and other markers located in the *purB* to *trpA* region were examined.

By making use of the $trpA_{amber}$ mutation in strain WGA and its derivatives the linkage between *narC* and the suppressor loci, *supC* (ochre) and *supIII* (amber), was determined by selecting for suppression of the *trp* mutation (Table 2). Interference from recombination at the *trp* locus was eliminated or made negligible by employing donors with *trp*− deletions or mutations.

Strain M226 containing the *galU*106 mutation of Shapiro (1966) was obtained as a source of the *galU* marker and the *narC* to *galU* linkage was established by transduction with phage *P1kc* (Table 2). During the course of these studies a curious property of *galU*− strains was revealed. At first it appeared that M226 was lysogenic for *P1* because it was resistant to phage *P1kc* but sensitive to *P1vir1* and lysates for transductions with M226 as donor had to be prepared with the latter phage. However, using these lysates with *P1*-sensitive recipients it was found that all *galU*− transductants were resistant to *P1kc* whereas all *gal*+ transductants from the same crosses were sensitive. Also, no phage could be detected

in sterile culture supernatants of M226 or derived *galU*$^-$ strains. Thus it appeared that the presence of *galU*$^-$ or a very closely-linked mutation was interfering with some stage in the infection and multiplication of the temperate phage. *GalU* is the structural gene for uridine diphosphoglucose pyrophosphorylase, and in addition to being unable to synthesize UDPG and ferment galactose, mutants are host-modifying for *T*-even phages due to defective glucosylation of phage DNA (Hattman and Fukasawa, 1963; Shedlovsky and Brenner, 1963) and they also fail to incorporate glucose or galactose into cell wall polysaccharides (Sundararajan, Rapin and Kalckar, 1962). The effect of the latter on the phage receptor sites was thought to be the most plausible explanation for resistance to phage *P1kc*. However, this might be expected to affect the susceptibility of *galU*$^-$ hosts to the virulent mutant *P1vir1* and also their transducibility by *P1kc*. In fact, *galU*$^-$ strains were sensitive to *P1vir1* and transduction frequencies with *galU*$^-$ recipients were generally low but no lower than is often obtained with many *galU*$^+$ recipients. The average frequency of *galU* transduction in 10 crosses involving *galU*$^-$ derivatives of W3110 as recipients was 23 *galU*$^+$ per 10^6 *P1kc* which is only 25% of the highest frequencies ever obtained. In a like number of crosses with M226 as recipient an average frequency of 8 *galU*$^+$ per 10^6 *P1kc* was found.

The *hemA* locus was recently located in the *purB-trp* region by Sasarman, Surdeanu and Horodniceanu (1968) and the present studies indicate that *narC* is closer to *hemA* than *purB* (Table 2). During the course of this work the linkages between other marker pairs were determined; average values are given in Fig. 2 and typical crosses are recorded in Table 3. The results are consistent with the marker order shown in Fig. 2 but they do not specify the position of *narC* relative to *galU* and the suppressor loci. The cotransduction frequency for *hemA* and *trp* (16—21%) indicates closer linkage than the 6% cotransduction reported by Sasarman *et al.* (1968); no previous estimates of the other distances have been reported.

Table 3. *Linkage relationships between markers in the purB-cysB region*

Donor (*P1kc*)	Recipient	Selective marker	Transductants per 10^6 phage	Number scored	Inheritance of non-selective donor markers	
					Marker	Linkage (%)
W	M226	*trp*$^+$	9.1	338	*galU*$^+$	66
W	M226	*galU*$^+$	11	149	*trp*$^+$	67
S729	WA-C31	*trp*$^+$	33	205	*hemA*$^-$	16
WA-C31	S729	*hemA*$^+$	71	206	*trpA*$^-$	21
S729	W-C31 *galU*$^-$[a]	*galU*$^+$	22	140	*hemA*$^-$	35
0254 *gal*$^+$	M226	*galU*$^+$	7.3	98	*purB*$^-$	2
W-C38	S730 *gal*$^+$*trp*$^+$	*hemA*$^+$	7.7	126	*purB*$^+$	6.4
S729	0254 *gal*$^+$*trp*$^+$	*purB*$^+$	2.3	150	*hemA*$^-$	4.0

[a] To obtain this recipient W-C38 was transduced to *nar*$^+$ by *P1vir1* lysates of M226 and a *galU*$^-$ transductant (*WgalU*$^-$) served as donor (*P1vir1*) for replacing the *galU*$^+$*trpA*$^-$ region of WA-C31 by *galU*$^-$*trpA*$^+$.

Table 4. *Three-factor crosses*

Cross	Donor	Recipient	Selective marker	Non-selective donor markers		Transductants per 10^6 phage	Number tested	Per cent transductants scored as				Indicated marker order
				A	B			AB	Ab	aB	ab	
1	W	WGAC-C49	*trp*$^+$	*nar*$^+$	*cys*$^+$	15	257	28	16	38	18	*narC-trpA-cysB*
			cys$^+$	*nar*$^+$	*trp*$^+$	26	131	10	3	54	33	
2	W-C38	M226	*gal*$^+$	*nar*$^-$	*trp*$^+$	10	120	48	29	16	7	*narC-galU-trp*
			trp$^+$	*nar*$^-$	*gal*$^+$	8.3	172	56	1.2	14	29	
3	W-C38	S730*gal*$^+$*trp*$^+$	*pur*$^+$	*nar*$^-$	*hem*$^+$	7.7	126	2	0	5	93	*purB-hemA-narC*
4	S729	WA-C31	*trp*$^+$	*hem*$^-$	*nar*$^+$	33	205	14.5	1.5	21	63	*hemA-narC-trpA*
5	S729	W-C31*galU*$^-$	*nar*$^+$	*hem*$^-$	*gal*$^+$	30	90	31	10	42	17	*hemA-narC-galU*
			gal$^+$	*hem*$^-$	*nar*$^+$	22	140	32	3	39	26	

A and B represent the non-selective markers of donor strains irrespective of whether they are wild-type or mutant alleles and a and b refer to the corresponding and alternate alleles of the recipient.

Three Factor Crosses. The position of the *narC* locus was confirmed by three factor crosses (Table 4). These indicate that *narC* is to the left of *trpA* and *galU* (crosses 1 and 2), to the right of *hemA* (crosses 3 and 4) and the order *hemA* .... *narC* .... *galU* is confirmed in cross 5. All the results are consistent with the order of markers shown in Fig. 2, but they do not specify the position of *narC* relative to the suppressor loci. This is only established by the studies with *Φ 80* (see below) and the known order of *galU* relative to the suppressors.

Fine Structure Analysis with narC Mutants. In order to investigate the genetic basis for the graded biochemical properties of *narC* mutants, selected strains were crossed in pairs. For this purpose *galU*$^-$ derivatives of the two mutants with differing phenotypes (C31 and C38) were prepared for use as recipients, and mutants representing the range of gas producing capacities were used as donors (all *gal*$^+$*trp*$^+$). The recombination frequencies for *nar*$^+$ relative to *gal*$^+$ (map distances) were low and in all cases the *narC* to *galU* linkages were significantly lower than normal indicating that the donor loci are situated between the recipient locus and *galU* (Table 5). On the basis of these results a genetic map of the *narC* region has been constructed (Fig. 3). No recombination between C38 (low gas producing) and C31 or C55 (high gas producers) was observed, but the resolution

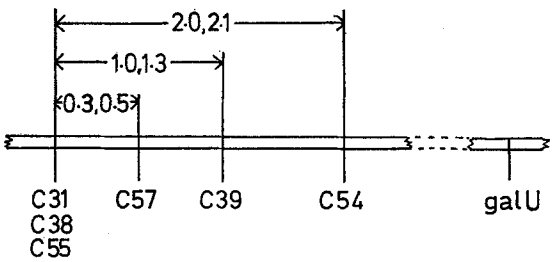

Fig. 3. Genetic map showing the positions of *narC* loci relative to *galU* based on the data given in Table 5

Table 5. *Fine structure analysis with narC mutants*

Donor	Recipient: M226-C38 *trp*$^+$				Recipient: W-C31 *galU*$^-$			
	Transductants per 10^8 *P1kc*		Map distance	Linkage (%)	Transductants per 10^8 *P1kc*		Map distance	Linkage (%)
	gal$^+$	*nar*$^+$			*gal*$^+$	*nar*$^+$		
W	630	83	—	63 (98)	2,235	2,230	—	68 (90)
W-C31	560	<0.2	<0.04	—	1,380	0.5	0.03	—
W-C38	590	<0.2	<0.03	—	1,180	<0.5	<0.04	—
W-C39	1,050	14.0	1.33	21 (62)	3,375	32.8	0.97	15 (91)
W-C54	270	5.5	2.04	18 (44)	1,040	21.5	2.07	16 (43)
W-C55	1,650	<0.2	<0.01	—	3,430	<0.5	<0.01	—
W-C57	700	2.2	0.31	18 (39)	1,510	7.0	0.46	8 (49)

Map distance equals the number of *nar*$^+$ transductants relative to *gal*$^+$ expressed as a percentage. Linkages were determined by scoring the distribution of the donor *galU*$^+$ marker in the *narC*$^+$ transductants (the number of transductants scored is in parenthesis).

was limited by poor transducibility of the *galU*$^-$ recipients. Also, no abortive transductants could be detected in any of the crosses but they may have been obscured by the rather heavy backgrounds caused by the necessity to plate a large number of recipient bacteria.

Transduction with Phage Φ80

The studies with phage *P1* show that *narC* is closest to the *supC*, *supIII* and *galU* genes, all of which are transduced by *Φ80*. Many attempts to transduce several *narC* mutants with induced lysates of *Φ80* have been made and although *trp*$^+$ and *galU*$^+$ transduction at frequencies of $2—8\times10^{-7}$ transductants/phage were observed, *narC*$^+$ transductants were never found ($<2\times10^{-10}$/phage), nor was joint transduction of *narC* observed with *gal* or *trp* as selective markers. Further attempts to transduce *narC* with high frequency transducing lysates from heterogenotes lysogenic for *Φ80* and *Φ80dgalU*$^+$, *Φ80dsupIII*$^+$*galU*$^+$ or *Φ80dsupC*$^+$*galU*$^+$ also failed. Thus it is concluded that *narC* is situated outside the region which is transduced by *Φ80*.

Discussion

Despite the variety of phenotypes exhibited by the *narC* strains they seem to arise by single mutational events which are clustered in the *hemA* to *supIII, C* region of the chromosome. This places *narC* close to *attΦ80* but apparently not close enough to be transduced by *Φ80*. Nevertheless the initial observation that related genes map near *attλ* and *attΦ80* may be significant.

The majority of *narC* mutants lacked only nitrate reductase activities. In this respect they differ from other nitrate reductase mutants which lack formate : nitrate oxidoreductase (but not necessarily BVH : nitrate oxidoreductase e.g. *narD*) and have additional deficiences in one or more components of formate hydrogenylase. Consequently, *narC* could be the structural gene for the nitrate reductase enzyme protein. If this is true, some explanation is necessary for the pleiotropic nature of the other *narC* mutants which also lack formate dehydrogenase and hydrogenase to varying extents.

One possibility is that in this minority class other genes specifying different components of the electron transport systems are affected. However, the tight clustering and the distribution of mutant loci representing the different phenotypes suggests that only one gene is involved. The apparent differences in *narC* mutants could then be explained if the nitrate reductase protein also participates in controlling the integrity of the enzyme complex. Thus some mutations may have a direct effect on its enzymatic function whereas others, in the same gene, may also affect its ability to associate with other proteins to form a functional unit. Consequently, formate dehydrogenase activity may be impaired and, in the absence of nitrate, uncoupling of hydrogenase may be apparent.

An alternative explanation depends upon the existence of a nitrate reductase operon containing several genes and including *narC*, the formate dehydrogenase gene and one specifying a component shared with the hydrogenase system. The pleiotropic nature of some of the *narC* strains could then be attributed to polarity mutations. According to this view, the C38 mutation would be of the extreme

polar or o°-type because it is defective in at least three respects and fine structure analysis indicates that it is situated at one extremity of the *narC* gene. Also in support of this possibility, mutations at internal sites, e.g. C39, should exhibit less polarity and this is apparent from the small effect on formate dehydrogenase and negligible effect on hydrogenase. This possibility is further supported by the recent findings of Ruiz-Herrera, Showe and DeMoss (1969) who have developed new methods for isolating strains which fail to reduce nitrate under normal growth conditions (NR^-). These mutants lacked formate dehydrogenase, nitrate reductase or various combinations of these activities and cytochrome b_1, and conjugation studies indicated that both formate dehydrogenase and nitrate reductase loci are linked to *trp*. The *narC* mutants are probably included in these NR^- strains, e.g. mutants like C31 may correspond to NR^- types 4 or 6 and C38 to types 7 or 8. None of the other NR^- types (2 and 5 which lack formate dehydrogenase but not nitrate reductase) would be expected amongst the *narC* mutants because they are probably anaerogenous and they may be sensitive to chlorate.

The choice of *galU* as an outside marker for ordering the *narC* loci was unfortunate in view of the associated resistance to *P1kc*. Since this work was completed the *galU* mutation has been shown to affect the adsorption of *P1kc* (Franklin, 1969). She observed 10- to 100-fold reductions in transduction frequency with $galU^-$ recipients, much greater than were found here. The apparent anomaly of transduction but lack of plaque-formation is explained by dependence of the former on a single event whereas the effects of impaired infection are compounded for the latter. Also, it is possible that fewer transduced bacteria may be lost as a result of lysis by superinfecting phage during selection. It must also be concluded that *P1vir1* resembles *P1clrg* (Franklin, 1969) in possessing an altered tail function permitting good adsorption to $galU^-$ hosts as well as being virulent.

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