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Genetics of Nitrofurazone Resistance in *Escherichia coli*

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Wild-type *Escherichia coli* cells are sensitive to nitrofurazone (NF) and many other nitrofuran derivatives. A variety of evidence indicated that these compounds are converted to toxic "active" metabolites by reductases present in the bacteria. Sensitive *E. coli* K-12 acquired threefold-greater resistance to NF in one mutational step. These partially resistant mutants could undergo a second mutation that made them 10 times as resistant as the wild type. Mutation of wild-type strain K-12 to the higher level of resistance in a single step was not observed. The first mutational step was associated with partial loss of reduced nicotinamide adenine dinucleotide phosphate-linked, O_2-insensitive NF reductase activity, and the second step was associated with loss of the remaining activity. The two-step mutants did, however, contain other NF reductases that were inhibited by O_2 and reduced NF only under anaerobic conditions. We designated the genes that control reductase activity "nitrofuran sensitivity genes" (*nfsA* and *nfsB*). Thus, wild-type strains are *nfsA*$^+$ *nfsB*$^+$, and the resistant double mutants are *nfsA nfsB*. A variety of crosses established that these genes are both located close to *gal*, that the most probable sequence is *lac nfsB gal nfsA*, and that the single-step mutants with an intermediate level of resistance are *nfsA nfsB*$^+$. The *nfsA*$^+$ *nfsB* strains contained about 70 to 80% of the wild-type reductase I activity—apparently enough to confer wild-type sensitivity. This reductase activity was resistant to 2 M urea. The *nfsA nfsB*$^+$ strains had only 20 to 30% of the wild-type activity, and this residual activity was sensitive to 2 M urea.

Since the work of Asnis et al., it has been known that nitrofuran derivatives are activated by flavoprotein reductases found in *Escherichia coli* and other bacteria (2, 3). Nitrofurazone (NF)-resistant mutants were shown to have lost a nitroreductase activity (reductase I) that is active in air but to have retained another nitroreductase activity (reductase II) that is active only under hypoxic conditions. Recently, we fractionated extracts of *E. coli* B/r on Sephadex G-100 and Sepharose 4B and showed that this strain contains at least three nitrofuran reductase components: reductase I (molecular weight, ca. 50,000) and two reductase II components (molecular weights, 120,000 and 760,000, respectively) (10). We have also shown that this strain mutates to nitrofuran resistance in two successive steps, the first of which is associated with partial loss of reductase I activity and the second with complete loss of this activity (11). No mutants that involve loss of reductase II activity have been isolated despite attempts to do so by treating suitable strains with high NF concentrations under anaerobic conditions (unpublished data).

Genes other than those that control reductase I are known to affect the response of *E. coli* to nitrofuran derivatives. Filamenting strains (*lon*) are about 20 times more sensitive to the lethal effects of these agents than are their nonfilamenting *lon*$^+$ (or *lon sul*) counterparts (9, 15). Mutations that cause defects in DNA repair (*uvr*, *lex*, and *recA*) also sensitize *E. coli* to killing by nitrofurans (8, 12, 16).

In this paper we report that there are two nitrofuran reductase I components in *E. coli* K-12 and that they are controlled by two different genes, both of which are linked to the galactose operon.

MATERIALS AND METHODS

Bacteria. *E. coli* K-12 strain AB1157 (F$^-$ *thr-1 leu-6 thi-1 supE44 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33* λ^- *sup-37*) (4) was obtained from the collection of the Medical Research Council Cell Mutation Unit. HfrH cultures were obtained from the Cell Mutation Unit collection and from Barbara Bachmann, Yale University, New Haven, Conn. The isolation and properties of resistant mutants are described in Table 1 and in Results.

Media and culture conditions. Bacteria were routinely cultured on nutrient agar (Oxoid, London, England) plates or in nutrient broth no. 2 (Oxoid) or Penassay broth (Difco Laboratories, Detroit, Mich.). Defined medium consisted of Davis-Mingioli medium

TABLE 1. *Strains used*

Designation	Parent(s)	Selective procedure	Phenotype[a]	Rate of whole-cell reduction in air[b]	Reductase I level	*nfs* genotype assigned[c]
AB1157	See reference 4		S	150	High	A^+B^+
NFR 402	AB1157	0- to 25-μg/ml gradient plate	I	50	Intermediate	AB^+
NFR 502	NFR 402	0- to 50-μg/ml gradient plate	R	0	Nil	*AB*
HfrH	See reference 4		S	190	High	A^+B^+
NFR 629[d]	HfrH	0- to 20-μg/ml gradient plate	I	23	NT[e]	AB^+
NFR 729[d]	NFR 629	0- to 40-μg/ml gradient plate	R	0	Nil	*AB*
SIL 28	Cross of NFR	See text	*gal*$^+$ NF-sensitive	NT	High	A^+B
SIL 7	729 ×		but mutated to re-	130	High	A^+B
SIL 46	AB1157		sistance in one	NT	High	A^+B
			step			

[a] S (sensitive) grows on 5 μg of NF per ml but not on 10 μg/ml; I (intermediate) grows on 15 μg/ml but not on 20 μg/ml; R (resistant) grows on 25 and 35 μg/ml.

[b] Nanomoles of NF reduced per hour by 1 ml of cell suspension having an absorbance at 600 nm of 1.0 (ca. 3×10^8 cells per ml).

[c] Assigned in the course of the work described here.

[d] Retained the Hfr character.

[e] NT, Not tested.

(7) with appropriate supplements. Most crosses were carried out in broth by standard procedures (13). Exconjugants were selected on defined medium containing appropriate supplements, picked, grown in nutrient broth, and then purified by cloning on the same selective medium. Isolated colonies were then picked, grown in broth, and tested for nutritional markers and sensitivity to NF. When *his*$^+$ was used as the selected marker, the conjugants were mixed in broth, a few minutes was allowed for attachment, and the cells were gently filtered onto a membrane filter (Millipore Corp., Bedford, Mass.), which was then laid on a nutrient agar plate. This procedure substantially increased the number of *his*$^+$ exconjugants recovered.

Chemicals. NF (semicarbazone of 5-nitro-2-furaldehyde) was prepared by a standard method (14). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co., London, England, and streptomycin was from Boots, Nottingham, England.

Gradient plates. NF was dissolved in dimethyl sulfoxide at a concentration of 4 mg/ml and stored for up to 3 weeks at −20°C. Appropriate volumes were added to molten nutrient agar held at 50°C. Plates were poured immediately, protected from light, and used within 3 days. Gradient plates were prepared by pouring 15 ml of drug-containing medium into sloping dishes (9 cm, round). After this layer had solidified, the plates were set on a level surface, and equal volumes of drug-free nutrient agar were added. Twenty-four hours were allowed for diffusion before gradient plates were used. For selection of resistant mutants, about 10^8 bacteria were spread on the plates by using a glass spreader. The plates were incubated at 30°C for 2 days; the colonies were then picked and subcultured.

Determination of NF sensitivity. In the early stages of the work, cultures (10^8 cells per ml) were streaked on gradient plates by using sterile capillary tubes. In subsequent work, the degree of resistance was determined by spot testing overnight broth cultures on a series of nutrient agar plates containing 5, 10, 15, 25, and 35 μg of NF per ml. In some experiments, plates with 45 μg of NF per ml were also included. Cultures (5 to 10 μl) were spotted on the agar surface by using sterile capillary tubes. Plates containing 5 to 25 μg of NF per ml were incubated for 24 h at 37°C, whereas the 35- and 45-μg/ml plates were incubated for about 40 h.

Determination of NF reduction by cells and extracts. NF reduction was determined as described previously (11). Briefly, reduction by whole cells was determined by suspending bacteria in 0.067 M sodium phosphate buffer (pH 7.2) containing 0.05 M glucose and about 10 μg of NF per ml and then incubating the mixture at 37°C with vigorous aeration. At intervals, samples were taken, the cells were centrifuged out, and the absorbance at 375 nm (the absorption maximum of NF) was determined.

Reduction by cell extracts was measured by recording the change in absorbance at 375 nm of a reaction

mixture containing 32 μg (0.16 μmol) of NF, 0.1 mg of NADPH, 20 μmol of glucose 6-phosphate, 40 U of glucose 6-phosphate dehydrogenase, and enzyme, in a total volume of 3.2 ml of 0.067 M sodium phosphate buffer (pH 7.2).

RESULTS

Sensitivity tests. The maximum NF concentration at which a given strain would grow was found to depend in part on the density of the culture applied to the plate. Therefore, to obtain valid comparisons between strains, it was necessary to adhere rigorously to the procedure described above. Labor-saving techniques such as replica plating gave inconsistent results. No advantage was gained by substituting appropriately supplemented minimal medium for nutrient agar in NF test plates.

Isolation of mutant strains. Resistant mutants were isolated from gradient plates inoculated with overnight broth cultures (Table 1) or from the 10 to 30 colonies that appeared when 10^7 cells of sensitive strains such as AB1157 or HfrH were plated on nutrient agar containing 10 μg of NF per ml. Cultures derived from these colonies almost invariably grew at 15 μg of NF per ml but not at 25 μg/ml, and had considerably reduced levels of reductase I activity.

When these single-step mutants were in turn plated (10^8 per plate) on nutrient agar containing 25 μg of NF per ml, 15 to 85 colonies appeared. Cultures derived from these proved to be resistant to about 45 μg of NF per ml and to lack detectable reductase I activity. No mutants resistant to this high level of NF were ever induced from wild-type *E. coli* K-12 strains in a single mutational step.

We have termed the genes involved "nitrofuran sensitivity" (*nfs*) genes and designated the wild-type alleles as *nfsA*$^+$ and *nfsB*$^+$, since mutation to resistance involves loss of enzyme activity. From the mutation and recombination data, the genotype of the two-step resistant mutants must be *nfsA nfsB*. The experiments described below establish that first-step resistant mutants invariably involve mutation of only one of these genes. This gene has arbitrarily been designated *nfsA*.

None of the NF-resistant strains had acquired any additional nutritional requirements. All grew well on minimal glucose medium supplemented to meet the requirements of the parental strain. Strains derived from HfrH (e.g., NFR 729), like those isolated from strain B/r, used glutamic acid and nitrate as the sole source of carbon and nitrogen, respectively.

Genetic analysis. In the following sections, the terms "sensitive," "intermediate," and "resistant" are used to denote strains that were inhibited by 5 to 10, 15 to 20, and >35 μg of NF per ml.

Preliminary crosses of resistant mutants derived from strain AB1157 with a variety of Hfr donors indicated that both of the genes involved in NF sensitivity are located between *pro* and *his* and that the two *nfs* genes undergo recombination with each other. Confirmatory data were obtained from crosses with HfrH (Table 2). The point of origin of this strain is located at about 95 on the recalibrated map, and the chromosome is transferred anti-clockwise so that the marker genes used enter recipients in the order of *pro*, *lac*, *gal*, and *his* (5). However, although there must be four *nfs* genotypes in the progeny, only the three already known were found. The remaining phenotype could mimic one of the other phenotypes, or it could be lethal. Taken together, the results of the crosses shown in Table 2 are most easily explained if the fourth genotype confers a sensitive phenotype. The two crosses are nonreciprocal in the frequencies of different recombinant classes. In particular, there is a large discrepancy in the frequency of exconjugants having the intermediate phenotype and an overall excess of sensitive recombinants when the data from the two crosses are combined. The data suggest that the *nfsA*$^+$ *nfsB* genotype does not give increased nitrofuran resistance compared with the *nfsA*$^+$ *nfsB*$^+$ wild type. Thus, the frequency of intermediates (especially in cross 2 of Table 2) is decreased and the frequency of apparent sensi-

TABLE 2. *Result of crosses*[a]

Phenotype	Genotype[b]	Cross 1: HfrH (*nfsB*$^+$ *gal*$^+$ *nfsA*$^+$) × NFR 502 (*nfsB gal nfsA*)		Cross 2: NFR 729 (*nfsB gal*$^+$ *nfsA*) × AB1157 (*nfsB*$^+$ *gal nfsA*$^+$)	
		No.	%	No.	%
Resistant	*nfsAB*	24	12	105	42
Intermediate	*nfsAB*$^+$	96	46	34	14
Sensitive	*nfsA*$^+$*B*$^+$ and *nfsA*$^+$*B*	87	42	108	44

[a] Selected marker, *gal*$^+$.
[b] See text and subsequent crosses for details.

tives is elevated. Obviously, if *nfsA*$^+$ *nfsB* (designated "silent") is indistinguishable from wild types, the genotype of the single-step mutants having an intermediate level of resistance must be *nfsA nfsB*$^+$.

Three predictions follow from the suggestion that the *nfsA*$^+$ *nfsB* genotype is as sensitive to NF as is the wild type. These will be examined in turn.

(i) All of the single-step mutants selected directly with NF should be *nfsA nfsB*$^+$ and none of them should be *nfsA*$^+$ *nfsB*. This prediction was tested by crossing six independently selected single-step mutants of strain AB1157 with strain NFR 629 (a single-step mutant of HfrH). Neither sensitive nor resistant exconjugants were found. All of the 240 strains tested were intermediate in phenotype. This result is consistent with the prediction made above but could arise for other reasons: for example, the mutation rate of one of the *nfs* genes might be substantially greater than that of the other.

(ii) In contrast to the wild-type sensitive strains, the "silent" *nfsA*$^+$ *nfsB* mutant should mutate to a high level of resistance in a single step. Since, as noted above, a reasonable proportion of the sensitive exconjugants in cross 2 of Table 2 were expected to be *nfsA*$^+$ *nfsB*, 26 sensitive exconjugants from a similar cross (NFR 729 × AB1157) were grown in broth, and 0.1-ml samples were plated on agar containing 25 μg of NF per ml. Other samples of these cultures were diluted 10-fold and plated on agar containing 10 μg of NF per ml. On the basis of the results obtained, the exconjugants could be divided into two classes, one of which gave mutant colonies only on the 10-μg/ml plates. Further testing of these colonies showed that only an intermediate level of resistance had been acquired. The second class produced mutant colonies on both 10 and 25 μg of NF per ml. Further testing showed that bacteria from colonies that appeared on the 10-μg/ml plates were resistant to at least 25 μg/ml. Clearly, these must have been derived from exconjugants that carried one mutant *nfs* allele but remained phenotypically sensitive (i.e., by mutation of *nfsA*$^+$ *nfsB* to *nfsA nfsB*).

(iii) If the "silent" sensitive strains are really *nfsA*$^+$ *nfsB* and the intermediate strains are *nfsA nfsB*$^+$, crosses between these two types should yield recombinants that are resistant to high levels of NF. This was tested by crossing "silent" strains derived from cross NFR 729 × AB1157 (putative *nfsA*$^+$ *nfsB*) with NFR 629 (putative *nfsA nfsB*$^+$), using *his*$^+$ as the selected marker. Since 6 to 7% of the *his*$^+$ recombinants were highly resistant to NF, the presence of the *nfsA* gene in the "silent," phenotypically sensitive mutant was confirmed (Table 3).

We have thus established that *nfsA* and *nfsB* are separate genes, that both are linked to *gal*, and that the *nfsA*$^+$ *nfsB* genotype does not confer increased resistance to NF. The determination of a definitive gene order was made more difficult by the fact that the *nfsB* gene is expressed only in the presence of *nfsA*. The following six gene orders are theoretically possible: (1) *pro nfsB nfsA gal*, (2) *pro nfsA nfsB gal*, (3) *pro nfsA gal nfsB*, (4) *pro nfsB gal nfsA*, (5) *pro gal nfsA nfsB*, and (6) *pro gal nfsB nfsA*.

A time-of-entry experiment (13) indicated that order 4 was most probable. Since it was not possible to select intermediate and sensitive exconjugants directly, *pro*$^+$, *lac*$^+$, and *gal*$^+$ were used as selective markers, and the phenotypes of a sample of the strains thus obtained were then determined as described above. The results (Table 4) show that *nfsB*$^+$ entered before *gal*$^+$, since at 28 min 10 of the 99 *lac*$^+$ exconjugants were intermediate but none was *gal*$^+$. It also appears that *nfsA* is distal to *gal*, since the fraction of *gal*$^+$-sensitive (*nfsA*$^+$ *nfsB*$^+$) exconjugants increased between 31 and 36 min. The phenotypically sensitive *gal*$^+$ colonies obtained at 36 min were examined to determine whether they were *nfsA*$^+$ *nfsB*$^+$ "sensitives" or *nfsA*$^+$ *nfsB* "silents." Of the 43 exconjugants, only one gave highly resistant mutants in a single step, indicating that the remaining 42 must have been *nfsA*$^+$ *nfsB*$^+$. The most likely gene order is thus no. 4. This order is consistent with the results

TABLE 3. *Results of cross NFR 629 (nfsB*$^+$ *gal*$^+$ *nfsA his*$^+$*) × silent (nfsB gal*$^+$ *nfsA*$^+$ *his)*[a]

Phenotype	Genotype	Cross 1 (SIL 28)		Cross 2 (SIL 46)		%
		No.	%	No.	%	
Resistant	*nfsAB*	4	3.4	9	9	6
Intermediate	*nfsAB*$^+$	24	24.5	11	11	16
Silent	*nfsA*$^+$*B*	89	76	48	49[b]	78
Sensitive	*nfsA*$^+$,*B*$^+$			30	31	

[a] Selected marker, *his*$^+$.

[b] These "silent" exconjugants mutated to "resistant" in a single step, whereas the sensitive strains mutated only to an "intermediate" level of resistance in one step (see text for further details).

TABLE 4. *Results of a time-of-entry experiment (HfrH × NFR 502)*

Entry of marker genes		Exconjugants					
Gene	Time of entry[a] (min)	Time of sampling (min)	Selected marker	NFR phenotype[b]			*gal*$^+$
				R	I	S	
pro$^+$	18	22	*pro*$^+$	100	0	0	ND[c]
lac$^+$	21	28	*lac*$^+$	89	10	0	ND
gal$^+$	29	31	*gal*$^+$	14	58	26	98
		36	*gal*$^+$	27	30	43	100

[a] From reference 5.
[b] R, Resistant; I, intermediate; S, sensitive.
[c] ND, Not determined.

TABLE 5. *Results of crosses*[a]

Phenotype	Genotype	Cross 1: HfrH (*pro*$^+$ *nfsB*$^+$ *gal*$^+$ *nfsA*$^+$) × NFR 502 (*pro nfsB gal nfsA*)				Cross 2: NFR 729 (*pro*$^+$ *nfsB gal*$^+$ *nfsA*) × AB1157 (*pro nfsB*$^+$ *gal nfsA*$^+$)		
		No.			%	No.		%
		a	b	c		a	b	
Resistant	*nfsA nfsB*	44	72	72	72.5	30	12	21
Intermediate	*nfsA nfsB*$^+$	19	6	12	14.3	2	5	3.5
Sensitive	*nfsA*$^+$ *nfsB*$^+$ and *nfsA*$^+$ *nfsB*	7	11	16	13.0	65	83	74.5

[a] Selected marker, *pro*$^+$.

shown in Table 2, since the frequency of intermediates in the cross HfrH × NFR 502 was greater than in cross NFR 729 × AB1157. This result would be predicted by the above order, since, in the first cross, intermediates would arise by a single crossover, whereas in the second a double crossover would be required.

Other data confirm this order. Table 2 eliminates orders 1 and 5, since double crossovers would be required to produce intermediates in the cross HfrH × NFR 502. Orders 2, 3, and 5 are also contradicted by Table 5. The frequency of intermediate resistance recombinants is higher in the cross HfrH × NFR 502 than in the cross NFR 729 × AB1157. This would be predicted by orders 1, 4, and 6, since in the second cross a double crossover would be required. By contrast, orders 2, 3, and 5 would predict the double crossover and lower frequency of intermediate resistance recombinants in the first cross. This leaves orders 4 and 6. Cross 1 (Table 5) also tends to favor order 4 and exclude order 6. Both of these orders predict that only a minority of sensitive recombinants will be "silent," so that *nfsB*$^+$ must be transferred in this cross at an appreciably higher frequency than *nfsA*$^+$.

Further evidence for order 4 comes from Table 6, which gives a detailed analysis of the association of *gal*$^+$ with nitrofuran resistance in the cross HfrH × NFR 502, selecting for *pro*$^+$. Salient points are the near absence of resistant *gal*$^+$ recombinants and the relatively high frequency of intermediate recombinants. These results tend to exclude orders 2 and 6 and fit order 4 best.

The results shown in Table 7, like the time-of-entry data, suggest that *nfsB* is located between *lac* and *gal*. The maximum possible number of exconjugants bearing *nfsB*$^+$ (the sum of the "intermediates" plus "sensitives") was 28 and may have been slightly lower since any *nfsA*$^+$ *nfsB* "silents" (which should have been rare relative to *nfsA*$^+$ *nfsB*$^+$ "sensitives") are included. In contrast, the number of *lac*$^+$ exconjugants was 47. Thus, the most probable order is: *lac nfsB gal nfsA*.

Relation of *nfs* genes to nitrofuran reductase. As noted in the introduction, *E. coli* B/r mutants having intermediate sensitivity to NF had lost a considerable amount of the O_2-insensitive NF reductase activity (reductase I), and in "resistant" mutants reductase I activity was undetectable (11). This situation was also found in the *E. coli* K-12 mutants used in this work; indeed, *nfsA nfsB*$^+$ strains contained only about 20 to 30% of the wild-type activity.

Chromatography of crude extracts from wild-type strains on Sephadex G-100 columns gave rather variable results but typically revealed a broad peak of NADPH-linked NF reductase activity, with a maximum corresponding to 56,000 daltons (Fig. 1). The proportion of the activity having a molecular weight of 56,000 increased when preparations were concentrated or aged. This suggests that the 56,000-molecular weight

TABLE 6. *Results of cross HfrH (pro⁺ nfsB⁺ gal⁺ nfsA⁺) × NFR 502 (pro nfsB gal nfsA)*[a]

Phenotype	Genotype	No.			%
		a	b	c	
Gal⁺ sensitive	*pro⁺ nfsB⁺ gal⁺ nfsA⁺* or *pro⁺ nfsB gal⁺ nfsA⁺*	4	8	13	9.6
Gal⁻ resistant	*pro⁺ nfsB gal nfsA*	44	71	72	72.2
Gal⁻ intermediate	*pro⁺ nfsB⁺ gal nfsA*	15	4	8	10.4
Gal⁺ intermediate	*pro⁺ nfsB⁺ gal⁺ nfsA*	4	2	4	3.9
Gal⁻ sensitive	*pro⁺ nfsB⁺ gal⁺ nfsA⁺* or *pro⁺ nfsB⁺ gal⁺ nfsA*	3	3	3	3.4
Gal⁺ resistant	*pro⁺ nfsB gal⁺ nfsA*	0	1	0	0.3

[a] Selected marker, *pro⁺*.

TABLE 7. *Results of cross HfrH (pro⁺ lac⁺ nfsB⁺ gal⁺ nfsA⁺) × NFR 502 (pro lac nfsB gal nfsA)*[a]

Phenotype	Genotype	No.
Resistant	*nfsA nfsB*	72
Intermediate	*nfsA nfsB⁺*	12
Sensitive	*nfsA⁺ nfsB⁺* and *nfsA⁺ nfsB*	16
Lac⁺	*lac⁺*	47
Gal⁺	*gal⁺*	17

[a] Selected marker, *pro⁺*.

peak is an aggregate and that the apparent lack of resolution may result from association-dissociation processes that take place during chromatography. Chromatograms of the extracts of the *nfsA⁺ nfsB* "silent" strains were similar to those of wild-type extracts in that they showed a broad peak with a maximum of activity at 56,000 daltons. A similar chromatographic analysis of the *nfsA nfsB⁺* (intermediate) strains showed a different pattern, with a maximum at around 38,000 daltons.

Further evidence that the enzymes controlled by the *nfsA⁺* and *nfsB⁺* genes represent distinct proteins was provided by experiments in which extracts from strain NFR 402 (*nfsA nfsB⁺*) and "silent" 7 (*nfsA⁺ nfsB*) were incubated with urea before and during the assay. The reductase component in NFR 402 (reductase 1b) was rapidly inactivated by 2 M urea (only 10 to 14% of the activity remained after 15 min), whereas the reductase (Ia) in "silent" 7 retained most of its activity for at least an hour.

As noted, *nfsA nfsB* strains still contain O_2-sensitive, NADPH-linked NF reductase activity (reductase II). Previous work with strain B/r showed that there are at least two reductase II components (11). More recent work (C. Lu and D. R. McCalla, unpublished data) has revealed a total of four such components.

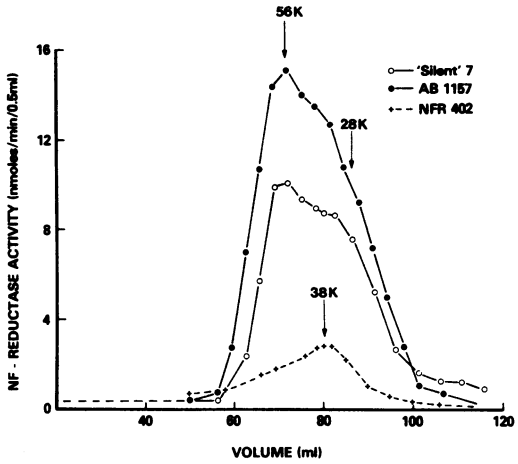

FIG. 1. *Chromatography of nitrofuran reductase I from E. coli on a Sephadex G-100 column (86 by 1.5 cm). Elution was with 0.067 M sodium phosphate buffer (pH 7.2). The activity was calculated by using the factor: ΔA_{375} of −0.05 represents reduction of 0.01 μmol of NF, where ΔA_{375} is the change in absorbance at 375 nm. The column was calibrated (1) with horse heart cytochrome c (molecular weight, 13,400), bovine pancreas chymotrypsin (molecular weight, 22,000), horse radish peroxidase (molecular weight, 40,000), and bovine serum albumin (molecular weight, 67,000). The apparent molecular weights of the reductase I components are indicated.*

DISCUSSION

The results presented above establish that two distinct genes (*nfsA⁺* and *nfsB⁺*) are involved in determining the sensitivity of wild-type *E. coli* K-12 to NF in air. Resistance to this agent and to many other nitrofuran derivatives (unpublished data) is acquired in two mutational steps. In the first step (*nfsA⁺* to *nfsA*),

a major fraction of the O_2-insensitive NF reductase (Ia) activity is lost while the second mutation decreases this enzyme activity below the level of detectability. Strains with the genotype *nfsA*$^+$ *nfsB* are not distinguishable fron the wild type in their sensitivity to NF. The two reductase components probably represent separate proteins, since they differ in their chromatographic properties and sensitivity to urea.

E. coli B/r, like strain K-12, acquires resistance to NF in two mutational steps (11), suggesting that the same two genes are involved. Strain WP 2, although it originated from B/r (6), acquires a high level of resistance to NF in a single mutational step (11; unpublished data). This suggests that WP 2 may have spontaneously acquired the *nfsB* mutation so that it is analogous to the "silent" (*nfsA*$^+$ *nfsB*) strains of K-12 constructed during this study.

It would be of considerable interest to know the "natural" substrate(s) and normal function of the NF reductases. Clearly these enzymes are dispensable under the growth conditions we employed. Indeed, as noted above, the NF-resistant strains do not seem to have any new nutritional requirements. We examined the possibility that the *nfs* genes might be identical to genes that have already been studied and found to map within a few units of *gal*. The obvious candidates (5) have been eliminated (unpublished data): nitrate reductase (*chlA chlD chlE*) on the basis of the ability of the NFR mutants to use nitrate as a N source and by direct tests on *chl* mutants for NF sensitivity; chloramphenicol resistance (*cmlA cmlB*) by direct tests on both *nfs* mutants and *cml* mutants (obtained from E. Reeves, University of Edinburgh); and succinic dehydrogenase (*sdh*) on the basis of use of glutamate as the sole source of N and C. Thus, at this stage, it would appear that the *nfs* genes are different from any previously described.

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