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Molecular cloning, characterization and expression of a nitrofuran reductase gene of *Escherichia coli*

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Abstract. Mini-mu derivatives carrying plasmid replicons can be used to clone genes *in vivo.* This method was adopted to generate phasmid clones which were later screened for their ability of restore nitrofurantoin sensitivity of a nitrofuran-resistant host by eliciting nitroreductase activity. One phasmid-derived clone (pAJ101) resulted in considerable increase in nitroreductase activity when introduced into a nitrofurantoin-resistant mutant of *Escherichia coli* with reduced nitroreductase activity. Subsequently, a 1·8 kb fragment obtained from pAJ101 by partial digestion with 5*au*3A, was subcloned into pUC18 to yield pAJ102. The nitroreductase activity attributable to pAJ102 was capable of reducing both nitrofurantoin and nitrofurazone. The polypeptides encoded by pAJ102 were identified by the minicell method. A large, well-defined band corresponding to 37 kDa and a smaller, less-defined band corresponding to 35 kDa were detected. Tnl000 mutagenesis was used to delineate the coding segment of the 1·8 kb insert of pAJ102. A 0·8 kb stretch of DNA was shown to be part of the nitroreductase gene. The gene was mapped at 19 min on the *Escherichia coli* linkage map.

Keywords. Molecular cloning; nitrofurans; nitrofuran reductase.

1. Introduction

Nitrofurans are synthetic antimicrobial agents extensively used in human and veterinary medicine (reviewed by Grunberg and Titsworth 1973). The discovery of antibacterial activity in the 5-nitro derivatives of some 2-substituted furans (Dodd and Stillman 1944) led to the development of a series of such compounds with varying degrees of biological activity, many of which were put to clinical use. Although the subsequent discovery of side-effects such as pulmonary toxicity (Taskinen *et al* 1977; Holmberg and Bowman 1981), hepatic damage (Goodman and Gillman 1975; Black *et al* 1980), peripheral neuritis (Paul and Paul 1964) etc., led to a re-evaluation of their chemotherapeutic effectiveness *vis-a-vis* safety, a number of compounds have remained in use. Nitrofurazone (as a topical antiseptic), nitrofurantoin (in urinary tract infections) and furazolidone (for gastrointestinal infections in cattle and poultry) are some of the nitrofurans still in use.

Central to the biological activity of these drugs is their metabolic activation by a class of enzymes called nitroreductases (reviewed by Kedderis and Miwa 1988). These enzymes activate nitrofurans by mediating the reduction of their nitro group. The existence of nitroreductases and the constitutivity of expression of the corresponding genes in many bacteria are obviously responsible for the broad

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spectrum of antibacterial activity of these drugs. However, the normal physiological role of these enzymes is not known. Despite the large body of evidence relating nitroreductase activity with nitrofuran sensitivity of bacteria, the individual steps in the mode of activation have not been studied in detail. Development of resistance to a nitrofuran is usually attributed to mutations which result in the loss of nitroreductase activity (Asnis 1957; McCalla *et al* 1970, 1975, 1978; Bryant *et al* 1981). In *Escherichia coli,* oxygen-sensitive (type II) and oxygen-insensitive (type I) nitroreductase activities have been identified (Asnis 1957; McCalla *et al* 1970, 1975, 1978). The oxygen-insensitive activity consists of major and minor components (McCalla *et al* 1978), the genes for which have been designated *nfs*A and *nfs*B, respectively. The minor nitroreductase activity has been shown to consist of two independent entities (Bryant *et al* 1981). The genes coding for the oxygen-insensitive nitroreductases have been broadly mapped to the *lac-gal* region (McCalla *et al* 1978). The precise map positions were however not identified. Two-step nitrofurazone-resistant mutants (see below) were shown to have lost the major nitroreductase activity and one of the two minor activities (McCalla *et al* 1978). Taking advantage of the correlation between nitrofurantoin resistance and loss of nitroreductase activity, we used a nitrofurantoin-resistant mutant as a host to isolate potential nitroreductase clones by screening for restoration of nitroreductase activity and drug sensitivity. In the present communication we report the cloning of a 1·8 kb fragment carrying the nitroreductase gene, identification of the encoded polypeptide and mapping of the cloned nitroreductase gene.

2. Materials and methods

2.1 *Bacterial strains and plasmids*

The *E. coli* strains and the plasmids used are listed in table 1.

2.2 *Chemicals*

All chemicals were obtained commercially and were of analytical grade. ^{35}S methionine was obtained from Amersham, England through Bhabha Atomic Research Centre, Bombay. ATP and NADPH were from Boehringer-Mannheim, Germany. Enzymes and other chemicals used during DNA manipulations were obtained from New England Biolabs, USA, Amersham, England and BRL, USA. The concentrations (μg/ml) of the antibiotics used were as follows: ampicillin, 100; tetracycline, 20; kanamycin, 40 and chloramphenicol, 6. Nitrofurantoin (Sigma Chemical Co., USA) was used at varying concentrations depending on the experiment.

2.3 *Isolation of nitrofurantoin resistant mutants*

Spontaneous nitrofurantoin-resistant mutants were isolated by plating approximately 10^7 cells of AB 1157 on L-plates containing 10 μg/ml of the drug and incubating the plates at 37°C. Putative mutants were patched on to fresh L-plates containing the same concentration of nitrofurantoin. The stability of the mutation was assessed by growing these mutants for several cycles in non-selective L-medium and plating

Table 1. Bacterial strains and plasmids.

Strain	Relevant genotype	Source/construction
Broda 8	*Hfr relA1 spoT1 metB1*	P Broda *via* B J Bachmann
HfrC	*Hfr relA1 spoT1*	L Cavalli–Sforza *via* B J Bachmann
KL208	*Hfr relA1? spoT1?*	K B Low *via* B J Bachmann
AB1157	F⁻ *thr1 leuB6 proA2 hisG4 argE3 thi1 rpsL31*	E A Adelberg *via* B J Bachmann
AB3027	F⁻ *thr1 leuB6 proA2 hisG4 argE3 thi1 polA20*	P Howard–Flanders *via* B J Bachmann
S1316	*araD139* △ *(argF-lac)* 169 *zbh-428::Tn10* *relA1 rpsL150* *bisC9::Mu cts deoC1?*	A Campbell *via* B J Bachmann
RW1230	△ *(gpt-proA)* 62 *zbj-1230::Tn10 hisG4 thi1*	R Kadner *via* B J Bachmann
MM383	*lacZ53 thyA36 rpsL151 polA12(Ts)*	M Monk *via* B J Bachmann
DS410	*azi-8? tonA2? minA1 minB2 rpsL135*	J N Reeve *via* B J Bachmann
RE103	*proA23 lac28 cmlA1 trp 30 his-51 rpsL101*	E C R Reeve *via* B J Bachmann
NFR502	AB1157 but *nfsA nfsB*	D R McCalla *via* B J Bachmann
Xph43 Mu *cts*	F⁻ △ *(argF-lac)U169 trp* △ *(brnQ phoA phoC phoB phoR)* Mu *cts*	M Casadaban
RJ101	△ *(lac-pro)* Rif^r *recA::Cm*	Laboratory collection
AJ101	One-step nitrofurantoin resistant mutant of AB1157	This work
AJ102	AJ101::Mu *cts*	This work
AJ212	Higher level nitrofurantoin resistant mutant (two-step mutant?) of AJ102	This work
AJ251	AB1157::Mu *cts*	This work
AJ301	MM383 with pAJ102 on the chromosome by integrative recombination	This work
AJ302	AB3027, Amp^r	This work
AJ321	MM383 with pAJ103 on the chromosome by integrative recombination	This work
AJ322	AB3027, Amp^r, Kan^r	This work
Plasmids:		
pEG5005	10·2-kb, pBCO::Mu d5005 Kan^r, Amp^r	M Casadaban
pUC18	2·7-kb, derivative of pBR322 Amp^r	J Messing
pKU602	4·6-kb, pUC18 carrying ApH gene from Tn5 Kan^r, Amp^r	K Dharmalingam
pAJ101	ca. 24-kb, phasmid-based nitroreductase clone, Kan^r	This work
pAJ102	4·5-kb, nitroreductase subclone, Amp^r	This work
pAJ103	6·4-kb, pAJ102 with Kan^r cartridge in nitroreductase gene Amp^r, Kan^r	This work

suitable dilutions on L-nitrofurantoin and L-streptomycin plates. A mutant (AJ101) which fulfilled the criterion of matching titres on both types of plates was lysogenized with Mu *cts*62 to give AJ102. A two-step mutant AJ212 was obtained from AJ102 as before but by spreading the cells on L-plates containing 70 μg/ml nitrofurantoin.

2.4 *Generation of phasmid library and screening for nitroreductase clones*

AB 1157 was lysogenized with Mu *cts*62 phage to give AJ251 which in turn was transformed to kanamycin resistance with pEG5005 isolated from Xph43Mu *cts* harbouring the phasmid. The lysate, obtained from pEG5005/AJ251 by heat induction, was used to transduce AJ212 to kanamycin resistance according to the protocol of Groisman and Casadaban (1986). The kanamycin-resistant transductants were screened for loss of ampicillin resistance to select for phasmids with an included chromosomal fragment (Groisman *et al* 1984). The kanamycin-resistant, ampicillin-sensitive transductants were gridded on to L-nitrofurantoin (70 and 30 μg/ml) and L-kanamycin plates. Since interpretation of nitrofurantoin resistance/sensitivity is often obscured by cell density, care was taken to make thin and broad patches while gridding. The transductants which showed no (or least) growth on L-nitrofurantoin plates were later assayed for nitroreductase activity.

2.5 *Nitroreductase assays*

2.5a *Reduction of nitrofurantoin by whole cells:* Strains were grown overnight in L-medium, diluted 1:100 in fresh medium and aerated at 30°C till mid-log phase. Appropriate antibiotics were included in the growth medium in the case of plasmid-bearing strains. The cells were collected, washed with M/15 phosphate buffer (pH 7·2) and the-absorbance at 600 nm was adjusted to 0·4. Glucose was added to 0·2% and the suspension was aerated at 30° C for 30 min. Nitrofurantoin was added to a concentration of 20 μg/ml and aeration continued. Three ml aliquots were removed at intervals, centrifuged in a microfuge for 3 min and the absorbance of the supernatant was measured at 372 nm (the absorption maximum of nitrofurantoin). Rate of nitrofurantoin reduction by whole cell suspensions was expressed as nmol reduced/ml. A decrease of 0·06 units in absorbance was taken to correspond to the reduction of 10 nmol of nitrofurantoin. Each value was obtained as an average of three independent experiments.

2.5b *Reduction of nitrofurantoin and nitrofurazone by cell free extracts*: The cells were grown as above, collected, washed with M/15 phosphate buffer (pH 7·2) and resuspended in the same buffer at 1/20 the original volume. The cells were lysed by sonication in a Labline ultrasonic system, with several 30s pulses at 100 W with 1-min intervals, the cells being kept cold on ice. The lysate was clarified at 100,000 *g* for 1 h at 4°C and the organelle-free supernatant was assayed for nitroreductase activity. The assay mixture contained: 300 μg NADPH, enzyme extract, nitrofurantoin (0·25 μmol) or nitrofurazone (0·3 µmol) and M/15 phosphate buffer (pH 7·2) in a total volume of 3 ml. The assay mixture was preincubated at 25° C without NADPH for 10 min and the reaction was initiated by the addition of NADPH. The reduction was monitored at 372 nm (nitrofurantoin) or 375 nm (nitrofurazone). The amount of drug reduced was calculated as follows: reduction of 10 nmol of nitrofurantoin and nitrofurazone results in a decrease of 0·06 and 0·05, respectively, at the respective absorption maximum. Each value was obtained as the average of three independent experiments. Protein was estimated by the method of Lowry *et al* (1951). Reductase activity was expressed as nmol of nitrofuran reduced/min/mg protein.

2.6 *DNA manipulations*

DNA manipulations were carried out according to the protocols outlined by Silhavy *et al* (1984). Subcloning of the nitroreductase gene was accomplished by digesting pAJ101 (see 'results') with different concentrations of *Sau*3A and ligating the digest containing the maximum number of fragments in the 1·8–2·4 kb range, into the *Bam*HI site of pUC18. The ligated mixture was used to transform AJ212 to ampicillin resistance.

Cartridge mutagenesis with the 1·9 kb *Bam*HI fragment from pKU602, carrying the kanamycin resistance gene, was performed by ligating the *Bam*HI fragment into the *Bg1*II site within the nitroreductase gene (see 'results').

2.7 *Genetic techniques*

P1 transductions and conjugational crosses were carried out according to Miller (1972) and Silhavy *et al* (1984), respectively. Integrative recombination of pAJ102 and pAJ103 was achieved essentially by the protocol of Cunningham and Weiss (1985). However, immediately after transformation the plates were incubated at 30° C. The transformants were pooled and grown in L-medium for a few generations at 30° C before the culture was subjected to alternating temperature shifts from 30° to 42° C and *vice versa.*

2.8 *Mutagenesis with Tn1000*

RJ101 carrying F'128 was transformed to ampicillin resistance with pAJ102. One of the transformants was used as donor in a cross with AJ212 (as recipient) and ampicillin-resistant transconjugants were obtained. The ampicillin-resistant transconjugants were screened for nitrofurantoin sensitivity/resistance by gridding on L-nitrofurantoin plates (30 μg/ml) and were also subjected to restriction analysis (see 'results').

2.9 *Identification of plasmid-encoded polypeptides*

Plasmid-encoded polypeptides were identified by the minicell method of Komai *et al* (1982). The minicell-producing strain DS410 was transformed to ampicillin resistance with pUC18 (control) and pAJ102 separately. Minicells were isolated on discontinuous sucrose gradients and labelled with ^{35}S methionine. The labeled minicells were mixed with sample buffer, kept in a boiling water bath for 3 min and the proteins were electrophoresed according to Laemmli (1970). The gel was fluorographed with PPO as the scintillant according to the method of Bonner and Laskey (1974), dried under vaccum and exposed to Indu X-ray film at – 70°C.

2.10 *Monitoring the response of AJ212 and AJ212/pAJ102 to nitrofurantoin challenge*

Overnight cultures of AJ212 in L-medium, and AJ212 harbouring pAJ102, in L-medium containing ampicillin, were diluted 1:100 in fresh L-medium and grown to a density of approximately 5×10^7 cells/ml. Nitrofurantoin was added to both cultures at 20 μg/ml and aeration continued. The kinetics of killing was monitored

by removing aliquots at 30-min intervals and plating suitable dilutions on L-plates. The plates were incubated at 30° C for 24 h to score for the survivors.

3. Results

3.1 *Isolation of nitrofurantoin-resistant mutants*

A stable mutant (AJ101) which satisfied the criterion of matching titres on media with and without nitrofurantoin was isolated as described under experimental procedures. It was found to tolerate up to 40 μg/ml nitrofurantoin although it was isolated on an L-plate containing 10 μg/ml nitrofurantoin. AJ101 was lysogenized with Mu *cts*62 to give AJ102. Whole cell suspensions of AJ102 displayed very low levels of nitroreductase activity (figure 1). Subsequently, a two-step mutant, AJ212, was isolated from AJ102. However, in the case of the two-step mutant, the high level of resistance was not stably maintained. Immediately after isolation the strain tolerated the drug up to a concentration of 100 μg/ml but on prolonged storage followed by growth in drug-free media the level of resistance dropped considerably. In any case, the level of resistance was greater than that of the parent strain, AJ101. The nitroreductase levels of the one- and two-step mutants were however the same (figure 1). In the light of these observations the nature of the second level nitrofurantoin resistance mutation in AJ212 is uncertain.

3.2 *Cloning of the nitroreductase gene*

The cloning strategy involved the generation of a phasmid-based library of a

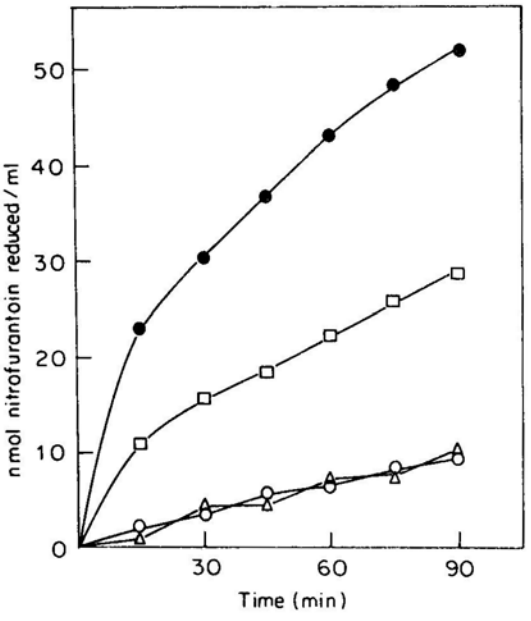

Figure 1. Nitroreductase activities of whole-cell suspensions.
(●), AJ212 carrying pAJ102;(□)AJ212 carrying pAJl0l; (O), AJ102;(Δ)AJ212.

nitrofuran-sensitive strain, transforming a nitroreductase deficient, nitrofurantoin-resistant strain and selecting a clone in which nitroreductase levels and drug sensitivity are restored. The phasmid-based clones obtained as described under experimental procedures were used to transduce AJ212 to kanamycin resistance. Approximately 5000 kanamycin-resistant transductants were screened initially for sensitivity to ampicillin and the ampicillin-sensitive clones were screened for nitrofurantoin sensitivity. Ten potential nitroreductase clones were isolated based on the extent of their growth on L-plates containing 70 and 30 μg/ml of nitrofurantoin and the levels of nitroreductase activity in whole-cell suspensions. However, only one of the isolates displayed stable nitrofurantoin sensitivity. The presence of the phasmid (pAJl0l) in this clone resulted in a substantial increase in nitroreductase activity in whole cell suspensions (figure 1). There was a corresponding increase in the activity in cell-free,extracts (table 2).

3.3 *Subcloning and restriction analysis of the nitroreductase gene*

The nitroreductase gene was subcloned into pUC18. In an earlier report by McCalla *et al* (1978) the sizes of the major and minor nitroreductases were estimated to be 56 and 38 kDa respectively. Therefore random fragments in the 1·8–2·4 kb size range, obtained by partial digestion of pAJl0l DNA with *Sau*3A, were ligated with *Bam*HI linearized pUC18 and used to transform AJ212 to ampicillin resistance. The transformants were screened for nitrofurantoin sensitivity and assayed for nitroreductase activity. One of the transformants displayed enhanced nitroreductase activity and carried a recombinant plasmid (pAJ102) with a 1·8 kb insert. Assays of the nitroreductase activities attributable to pAJl0l, pAJ102 and the mutant host AJ212, both *in vivo* (figure 1) and *in vitro* (table 2) clearly demonstrate that pAJ102 includes the nitroreductase gene. The higher level of reductase activity due to pAJ102 can be attributed to the copy number of the plasmid. The possession of pAJ102, renders the cell hypersensitive to nitrofurantoin (see below). Since nitroreductases are known to activate a variety of nitrofurans, the activity was assayed with both nitrofurantoin and nitrofurazone as substrates (table 2). It is apparent that the activity is not restricted to nitrofurantoin but extends to atleast one other nitrofuran.

The presence of convenient restriction sites on the vector facilitated restriction analysis of the 1·8 kb insert. A partial restriction map of the 1·8 kb insert is depicted in figure 2a.

3.4 *Mutagenesis of pAJ102 with Tn1000* ($\gamma\delta$)

In order to delineate the segment coding for nitrofuran reductase within the 1·8 kb

Table 2. Nitrofuran reductase activities *in vitro.*

Source of enzyme extract	Specific activity[a] with nitrofurantoin	Specific activity[a] with nitrofurazone
AJ212	3·7	3·7
AJ212 harbouring pAJ101	463	564
AJ212 harbouring pAJ102	686	813

[a]nmol of drug reduced/min/mg protein.

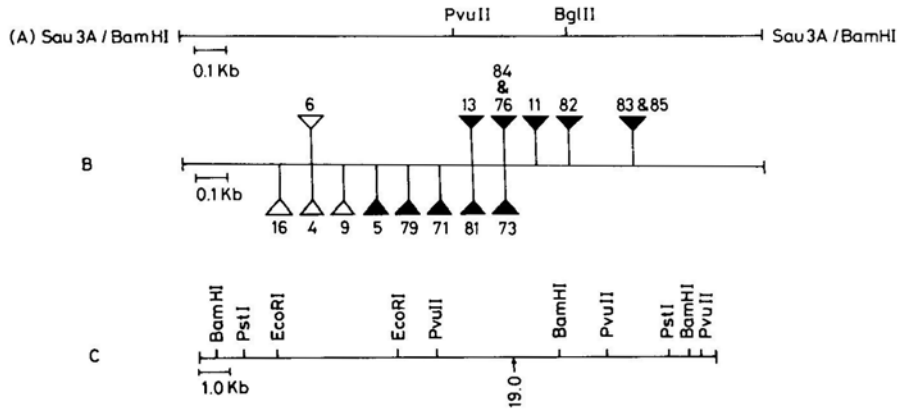

Figure 2. (**A**) Partial restriction map of insert in pAJ102.
(**B**)Location of Tnl000 on the 1·8 kb insert of pAJ102.
Tnl000 in *γδ* (∇,▼) and *δγ* (Δ,▲) rientation not affecting (∇,Δ) and inactivating (▼,▲) nitroreductase.
(**C**)Partial restriction map of the chromosomal insert in pAJl0l. The arrow indicates 19th min of the *E. coli* genetic map.

The left and right ends of the figure correspond to the *Hind*III and *Eco* RI ends of the multiple cloning site of *pUC*l8. Scale (**A**) and (**B**), 1 cm = 0·l kb. (**C**) 1 cm= 1·0 kb.

insert of pAJ102, Tnl000 mutagenesis was carried out. The plasmid was first introduced into an F'-bearing strain (RJ101) and subsequently mobilized into a nitrofurantoin-resistant recipient (AJ212) selecting for amp^r transconjugants. F-mediated mobilization of non-conjugal plasmids involves transposition of the *γδ* (Tnl000) element on to the plasmid, cointegrate formation, transfer through the F-conjugal system and resolution of the cointegrate in the recipient. Transposition of Tnl000 could result in insertional Inactivation of gene function. Since pAJ102 harbours an insert coding for nitroreductase, insertions into the coding segments could be picked up by screening for loss of enzyme activity. The recipient (AJ212) is deficient in nitroreductase and is resistant to nitrofurantoin (see above). Introduction of a plasmid containing an insertionally-inactivated nitroreductase gene would not affect the resistance status of the host while introduction of a functional gene would. Screening for the persistence of nitrofuran resistance of the recipient would allow the detection of clones inheriting the insertionally- inactivated gene. However, loss of resistance would not distinguish between insertions on the vector and those in regions of the insert not coding for nitroreductase. Therefore the amp^r transconjugants were also subjected to restriction analysis which revealed sixteen insertions within the 1·8 kb insert of pAJ102. Using the known restriction maps of pUC18 and Tnl000 the orientations of Tnl000 in each of the insertions were also determined. Figure 2b presents the data. It can be seen from figure 2b that Tnl000 insertions in 0·5 kb of DNA at the left end of the 1·8 kb insert do not inactivate the nitroreductase gene (insertion nos 4, 6, 9 and 16). Insertions in the next 0·8 kb of DNA (nos 5, 11, 13, 71, 73, 76, 79, 81, 82, 83, 84 and 85) inactivated the gene. For reasons not known we did not get insertions in the last 0·5 kb of the insert. Therefore the maximum stretch of DNA available in the 1·8 kb insert for encoding the nitroreductase enzyme is 1·3 kb. Accordingly the size of the encoded

polypeptide could be approximately 40 kDa. Superimposition of the partial restriction map of the 1·8 kb insert and the map of the Tn1000 insertions into the same clearly shows the inclusion of the *Pvu*II and *Bgl*II sites within the coding region.

3.5 *Identification of plasmid-encoded polypeptides*

The minicell producing strain DS410 was transformed to ampicillin resistance with pUC18 (control) and pAJ102. The minicells were isolated on discontinuous sucrose gradients, labelled with ^{35}S methionine and the proteins were solubilized with sample buffer. The plasmid-encoded polypeptides were identified by SDS-PAGE followed by autoradiography (figure 3). Two additional polypeptides were observed in minicells harbouring pAJ102 (figure 3, lane 2): a major band corresponding to 37 kDa and a minor band corresponding to 35 kDa. The major band was well-defined whereas the minor band was somewhat fuzzy and ill-defined. Considering the stretch of DNA in the 1·8 kb insert identified to constitute the nitroreductase gene (described above), the 37 kDa polypeptide should be the nitroreductase protein while the 35 kDa polypeptide can be attributed to degradation or processing of the 37 kDa polypeptide.

3.6 *Kinetics of survival of AJ212 and AJ212 harbouring pAJ102 upon nitrofurantoin challenge*

Since there is a direct correlation between nitrofuran reductase activity and nitrofuran sensitivity, an enhancement in nitroreductase activity would lead to increased nitrofuran sensitivity. The effect of nitrofurantoin on the viability of AJ212 (control) and AJ212 harbouring pAJ102 was monitored at different time intervals after exposure to nitrofurantoin. At a concentration of 20 μg/ml, nitrofurantoin drastically affected the survival of AJ212 harbouring the nitroreductase clone (figure 4). Within 30 min of addition of the drug, the number of survivors decreased more than 100-fold. (At earlier intervals, there was inhibition of colony-forming ability. On prolonged incubation in the absence of the drug, cells did grow to form colonies). In the case of normal, plasmid-free, nitrofuran-sensitive cells it takes approximately 7 h to obtain the same level of killing under identical conditions (D N Simha and R Jayaraman, unpublished results). The presence of pAJ102 did not affect the growth of AJ212 in the absence of nitrofurantoin (data not shown).

3.7 *Mapping of the nitroreductase gene*

A previous report (McCalla *et al* 1978) had indicated only the approximate map locations of the nitroreductase genes. In order to map the location of the gene that we had cloned, two approaches were followed. In one, the plasmid carrying the cloned gene was forced to integrate into the chromosome. This resulted in the transfer of plasmid-borne drug resistance marker to a chromosomal locus, homologous to the insert carried on the plasmid. The drug resistance marker was then utilized to map the locus of integration by conventional methods. Since DNA polymerase I is essential for the replication and maintenance of *col*EI replicons,

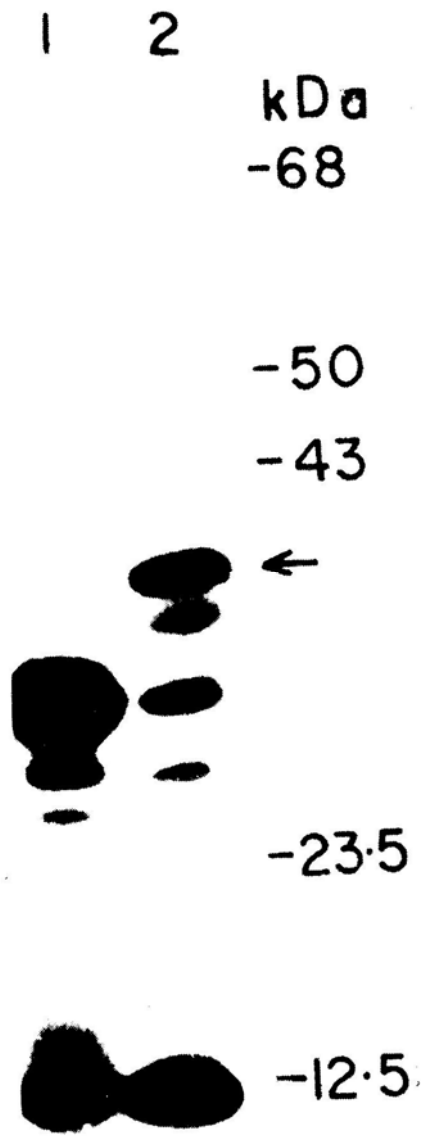

Figure 3. Detection of plasmid-encoded polypeptides by the minicell method. DS410 was transformed separately with pUC18 (control) and pAJ102. The minicells were isolated on discontinuous sucrose gradients and labelled with ^{35}S methionine. The plasmid-encoded polypeptides were identified by PAGE followed by autoradiography.
Lane 1: pUC18 encoded polypeptides. Lane 2: pAJ102 encoded polypeptides. Arrow indicates position corresponding to 37 kDa.

selection for drug resistance under conditions which rendered the enzyme inactive would yield derivatives with the plasmid integrated into the chromosome. Towards this end a *pol*A ts strain (MM383) was transformed with pAJ102 and the ampicillin-resistant transformants were subjected to alternate cycles of growth at 30° and 42° C. In another experiment a drug resistance marker was first introduced into the

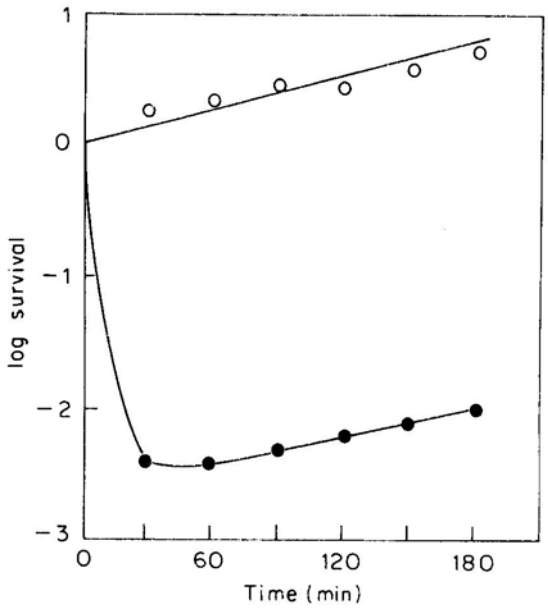

Figure 4. Response of AJ212 and AJ212 harbouring pAJ102, to nitrofurantoin challenge. (O), AJ212; (●), AJ212 harbouring pAJ102.

nitroreductase gene as follows. Since the *Bgl*II site lies within the gene (see above) a 1·9 kb *Bam*HI fragment from pKU602 carrying the gene for kanamycin resistance was ligated into it (cartridge mutagenesis). The resultant plasmid pAJ103 was used to transform MM383. Chromosomal integration of pAJ103 was achieved as described above for pAJ102.

The kanamycin and ampicillin resistance markers (due to integrated pAJ103 and pAJ102) from two derivatives of MM383 (AJ321 and AJ301), were transduced into AB3027. The nitroreductase activities of whole-cell suspensions of a kanamycin resistant transductant (AJ322) and an ampicillin-resistant transductant (AJ302) were determined as before. It is apparent from figure 5 that integration of pAJ102 led to a slight increase in nitroreductase activity whereas integration of pAJ103 did not. This can be explained by the fact that integration of pAJ102 which has an intact nitroreductase gene would give rise to two functional copies of the gene whereas integration of pAJ103 would still have only one functional copy of the gene. The duplication of the nitroreductase gene as a result of integration of pAJ102 leads to an increase in nitroreductase activity (figure 5).

Conjugational crosses between AJ322 (as the recipient) and various Hfrs showed that a good proportion of gal^+ recombinants lost kanamycin resistance (table 3) suggesting that the cloned gene could lie in the vicinity of the *gal* locus. Subsequently, the kanamycin resistance marker of pAJ103 and the ampicillin resistance marker of pAJ102 were mapped by transduction of tetracycline resistance from strains carrying Tnl0 insertions close to the *gal* locus and screening the tet^r transductants for the loss of kanamycin and ampicillin resistance, respectively. Reciprocally, the kanamycin resistance marker of AJ322 was transduced into RW1230 and the kan^r transductants were screened for loss of tetracycline resistance.

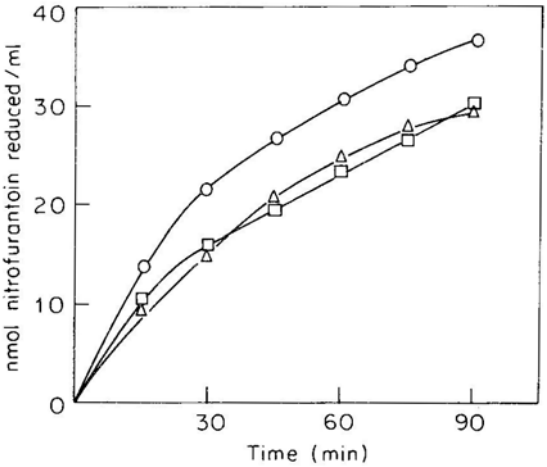

Figure 5. Nitroreductase activities of whole-cell suspensions of AJ302(O), AB3027(Δ) and AJ322(□).

Table 3. Conjugational crosses between AJ322 and various Hfrs.

Donor	Origin (min) and direction of transfer	Selected marker	Unselected marker	Unselected/ selected (%)
Broda 8	9·5/cc	His^+	Kan^s	10·4 (11/106)
HfrC	13/c	Pro^+	Kan^s	— (0/112)
HfrC	13/c	Thr^+ Leu^+	Kan^s	— (0/116)
Broda 8	9·5/cc	Gal^+	Kan^s	42·9 (72/168)
KL208	30/c	Gal^+	Kan^s	70·5 (93/132)

c: Clockwise; cc: counter clockwise.

Table 4. Transductional mapping of the nitroreductase gene.

Donor	Recipient	Selected marker	Unselected marker	Cotransduction frequency (%)
RW1230	AJ322	Tet^r	Kan^s	1·13 (13/1147)
S1316	AJ322	Tet^r	Kan^s	3·95 (14/354)
RW1230	AJ302	Tet^r	Amp^s	0·97 (5/517)
AJ322	RW1230	Kan^r	Tet^s	0·98 (8/813)
AJ322	RE103	Kan^r	Cml^s	98·19 (163/166)

The results of the transductional analyses are presented in table 4. It can be seen that the nitroreductase locus is cotransducible with the Tnl0 insertions in RW1230 and S1316 at a low frequency (1–4%). This observation suggests that the gene could lie between the loci of the two Tnl0 insertions (17–19 min). Calculation of the map distances using Wu's formula (Wu 1966; taking L = 2·3) would place the position of the nitroreductase gene between 18·5 and 19·1 min on the linkage map. Table 4 also shows that the nitroreductase locus is very highly cotransducible with *cml*A (18·8 min). Thus the transductional analyses suggest that the nitroreductase gene could be located at 18·8 min.

In the second approach the restriction profile of the chromosomal insert in pAJ101 (figure 2C) and the restriction map of the *E. coli* genome (Kohara *et al* 1987) in the region indicated by genetic methods were compared. The restriction map of the *E. coli* chromosome at a location close to 19 min (18·8–19·1 min; kilobase coordinates approximately 895 to 915) matches precisely with the restriction map of the insert in pAJ101. The gene can therefore be placed at 19 min. The *Pvu*II site within the nitroreductase gene facilitated the identification of the map position. The slight discrepancy between the map positions obtained by the two methods can be ascribed to the relative imprecision of conventional genetic methodologies.

4. Discussion

In this report we have presented data on the cloning and characterization of a gene from *E. coli* coding for nitrofuran reductase. Since nitrofuran resistance and nitroreductase activity bear an inverse correlation we isolated a nitrofurantoin-resistant mutant with low levels of nitroreductase activity and used this to screen phasmid-based clones for restoration of enzyme activity and drug sensitivity. A 1·8 kb fragment of the chromosomal insert of one such clone obtained this way (pAJ101) was subcloned into pUC18, to give pAJ102. The observation that the nitroreductase activity of the host strain was very low in whole cell suspensions as well as cell-free extracts showed that the mutation inactivated the nitroreductase gene rather than creating a simple permeability barrier to nitrofurantoin. The restoration of nitroreductase activity by pAJ101 and pAJ102 in whole cells as well as cell-free extracts showed that the plasmids contain the gene coding for the enzyme. Possession of pAJ101 and pAJ102 renders AJ212, a nitrofurantoin-resistant mutant, highly sensitive to the drug. Although an earlier report (Herrlich and Schweiger 1976) suggested that unreduced nitrofurantoin can also have biological activity, our results demonstrate the need for reductive activation.

The identity of the cloned gene with *nfs*A or *nfs*B genes defined by McCalla *et al* (1978) can be deduced from the size of the encoded polypeptide, map position of the gene and level of enzyme activity due to a single copy of the gene. From the size of the coding segment of the insert (≤1·3 kb, as determined by Tn1000 mutagenesis) and the size of the polypeptide (37 kDa) identified in minicells, it is probable that the cloned gene could be *nfs*B. However the map location of the gene is at variance with that reported by McCalla *et al* (1978). Although the authors had not reported precise map positions, they had suggested that the order of loci in the relevant region could be *lac* (8 min) *nfs*B (?) *gal*K (17 min) *nfs*A (?). That is, *nfs*B lies before 17 min on the *E. coli* linkage map. Our mapping data presented herein place the cloned gene at 19 min. However, a careful and critical analysis of the genetic data reported by McCalla *et al* (1978) does show that the more probable order could be *lac gal nfs*B *nfs*A. If this were so, *nfs*B would fall in the same position as reported herein. McCalla *et al* (1978) had isolated a two-step nitrofurazone-resistant mutant (NFR 502) which lacked both the major and minor nitroreductases. When this strain was transduced to kanamycin resistance using P1 propagated on AJ322 (see 'results') the nitroreductase activity was restored to only 10% of wild type levels (unpublished results). It could be recalled that AJ322 contains two copies of the nitroreductase gene, only one of which is functional and the other inactivated by

insertion of the kanamycin-resistant determinant. Transduction of kanamycin resistance using AJ322 as donor, in effect, introduces a single functional copy of the nitroreductase gene. The observation that enzyme activity is restored only to 10% of normal levels suggests that the cloned gene codes for the minor nitroreductase. The major and minor nitroreductases have been reported to differ in their sensitivity to 2 M urea. While McCalla *et al* (1978) showed that the minor enzyme was sensitive, Breeze and Obeseiki-Ebor (1983) have shown exactly the opposite. We have partially purified the enzyme encoded by the insert carried on pAJ102, by affinity chromatography on Sepharose CL-6B and found the partially purified enzyme to be relatively insensitive to urea (25% loss of activity after 45 min exposure to 2 M urea at 37°C; unpublished results). In view of the existence of mutually contradictory reports, urea sensitivity is not a satisfactory criterion to establish the identity of nitrofuran reductases. However, based on other criteria reported herein we believe that the gene we have cloned is *nfs*B. Thus, this work has established the precise map position of one of the genes involved in nitrofuran toxicity. Watanabe *et al* (1989, 1990) have reported the cloning of a nitroreductase gene of *Salmonella typhimurium.* The size of the corresponding polypeptide was found to be 28 kDa. Since several enzymes are known to be involved in the metabolism of nitroheterocycles, it is difficult to say whether the gene cloned by Watanabe *et al* (1989, 1990) is analogous to the one reported herein.

pAJ102 can be exploited as a cloning vector since insertion of extraneous DNA into the nitroreductase gene will inactivate it and thereby permit direct selection when transformed into a nitrofurantoin-resistant host. This is currently being assessed.

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