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Isolation of Single-Site *Escherichia coli* Mutants Deficient in Thiamine and 4-Thiouridine Syntheses: Identification of a *nuvC* Mutant

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A method is described to rapidly select and classify many independent near-UV irradiation-resistant *Escherichia coli* mutants, which include tRNA modification and RNA synthesis control mutants. One class of these mutants was found to be simultaneously deficient in thiamine biosynthesis and in the ability to modify uridine in tRNA to 4-thiouridine, known to be the target for near-UV irradiation. These mutants were found to be unable to make thiazole, a thiamine precursor. The addition of thiazole restores the thiamine deficiency but does not render the cells near-UV irradiation sensitive. In vitro studies on one of these mutants indicated a deficiency in protein factor C (*nuvC*), required for the 4-thiouridine modification of tRNA. In P1 transduction, the thiazole marker cotransduced with the histidine marker, which places the thiazole marker between 42 and 46 min on the *E. coli* chromosome map. Both thiamine production and 4-thiouridine production were resumed by 87% of the spontaneous reversions, suggesting a single-point mutation. Our results indicate that we have isolated *nuvC* mutants and that the *nuvC* polypeptide is involved in two functions, tRNA modification and thiazole biosynthesis.

Growth delay in *Escherichia coli* after irradiation with light in the near-UV range (340 nm) has been traced to the presence of 4-thiouridine (4-Srd) in the tRNA (6, 12). The 4-Srd residue in tRNA (at position 8) absorbs light of this wavelength and forms an adduct with a cytidine residue (at position 13 in the D-stem), thereby reducing the activity of some species of tRNA (14, 16). This results in amino acid starvation and reduced growth of the bacteria. When the near-UV irradiation ceases, the cells recover by de novo synthesis of active tRNA. In this manner, a pulse of near-UV irradiation causes a growth delay, i.e., inhibition plus resumption of growth.

Two types of near-UV irradiation-resistant mutants have been found (12, 14): (i) cells that lack the 4-Srd modification in tRNA and (ii) cells which are unable to accumulate high concentrations of guanosine tetraphosphate during amino acid starvation (Rel phenotype) and which therefore fail to show the so-called stringent response, a curtailment of rRNA and tRNA syntheses during amino acid starvation (15). The first type of mutant shows no growth delay after near-UV irradiation, since there is no target for induction of growth delay; the second type of mutant has a shorter growth delay, because of increased synthesis of new tRNA during the near-UV irradiation-induced amino acid starvation.

These two types of mutants were obtained by Ramabhadran (11) by subjecting cultures of *E. coli* bacteria (of the Rel^+ phenotype) to repeated cycles of near-UV irradiation and overnight growth; each cycle produced a 5- to 10-fold enrichment of the mutant over the parental phenotype. We report here the results of a modification of this procedure which allows one to isolate hundreds of independent mutations to near-UV resistance on a single plate.

With this method, it was surprisingly found that 25% of the mutants lacking the 4-Srd tRNA modification were also auxotrophic for thiamine, or rather for the thiazole portion of thiamine. Although the presence of thiazole in the growth medium relieved the requirement for thiamine, it did not restore the conversion of uridine to 4-Srd in tRNA. The site of this mutation lies near *his* rather than in the region of the known *thi* genes. Further study showed that these mutations were in *nuvC*, the structural gene for one of the two protein factors (factor C) required for the 4-Srd modification in tRNA (8). Mutations in this gene have not been previously reported (see below).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study and their sources are listed in Table 1. The following growth media were used: medium C (5) supplemented with either 0.2% glucose and 0.6% Difco Casamino Acids or 0.2% glucose only; LB medium (9); and M9 medium (9). Minimal agar plates and LB agar plates were prepared as described previously (9). Bacterial cultures were grown in a shaker at 30°C, and growth was followed by measuring the turbidity at a 460-nm wavelength (optical density at 460 nm, 1-cm light path). Agar plates were incubated at 30°C. Phage P1 transduction was carried out according to Miller (9).

Detection of 4-Srd in tRNA. The fluorescence emission and excitation spectra of sodium borohydride-reduced tRNA that had been near-UV irradiated in vivo was taken as an indication of the presence of 4-Srd in tRNA. The method used was described by Ramabhadran et al. (13). Briefly, 2-liter cultures of either *E. coli* RYH101 or *E. coli* B/r were grown to an optical density at 460 nm of 1.0 in medium C supplemented with 0.2% glucose and 4 μg of thiamine per ml and concentrated 10-fold by centrifugation, and one-half of the concentrated cultures was irradiated with near-UV irradiation with a fluence of 150 kJ/m^2. The cells were further concentrated and deproteinized by three phenol extractions. In each sample, the nucleic acids (mainly tRNA) were ethanol precipitated and finally treated with freshly prepared $NaBH_4$ at pH 9.7. The near-UV absorption spectra of the tRNA was recorded before and at various times after the addition of the $NaBH_4$ with a Cary 14 recording spectrophotometer.

The distribution pattern of sulfur among the various thionucleotides in tRNA from RYH101 was determined as previously described (8) by isolation of tRNA from cultures grown in 1 liter of M9 medium containing 1 mCi of carrier-free $H_2{}^{35}SO_4$, alkaline digestion to mononucleotides, dephosphorylation, and chromatography on cellulose phosphate columns.

In vitro measurement of thiolating activity. As previously described, extracts from alumina-ground cells were partially purified using $(NH_4)_2SO_4$ fractionation and a DEAE column to remove endogenous RNA (8). The thiolating activities of these extracts were measured by incubation with Mg^{2+}, ATP, [^{35}S]cysteine, 2-mercaptoethanol, *E. coli* tRNA (100 units of absorbancy at 260 nm) as the acceptor, and where indicated, supplementary factors A and C, purified from *E. coli* B. Details of the assay are published elsewhere (1).

RESULTS

Isolation of near-UV irradiation-resistant bacteria. To isolate near-UV irradiation-resistant mutants, a diluted overnight culture of *E. coli* NC51 in LB medium was plated on an LB agar plate (500 bacteria per plate); after 12 h of incubation at 30°C, microcolonies had grown with about 10^6 cells per colony. It was assumed that many of these microcolonies would have one or more bacteria with a spontaneous mutation to near-UV irradiation resistance. These mutant bacteria were given a selective advantage by further incubating the plate with intermittent near-UV irradiation. The plates were placed upside down on a glass plate suspended 2 cm over two parallel near-UV lamps (type FI5T8/BLB; Sylvania Corp.). The lamps were turned on and off with an automatic timer. The lamps were on for 1.5 h and off for 4.5 h (one cycle of light and dark). The dose at the dish surface was determined with a UV dosimeter (14; Weston Instruments) to be 32 ± 3 J/m^2 per s, and a fan was used to keep the temperature of the plates at 30°C. The biological effect of the lamps was always monitored by placing NC51 (near-UV irradiation-sensitive strain) and NC52 (near-UV irradiation-resistant strain) on separate plates at the area of weakest intensity. As a further control, one-half of each plate was shaded with cardboard. After 40 h of intermittent near-UV irradiation, the bacteria on the shaded (i.e., unirradiated) half had formed large-sized colonies, and the bacteria on the near-UV irradiated half either had formed intermediate-sized

TABLE 1. *E. coli* strains used

Strain	Genotype	Phenotype			Origin
		Thi	UV resistant[a]	tRNA modification[b]	
B/rA		+	−	+	ATCC 12407
CSH57	*ara lac leu purE gal trp his arg mal str xyl mtl ilv met*	+	ND	ND	Cold Spring Harbor Laboratory
JR100	*metA argH purF xyl supE rpoB*	+	ND	ND	Spontaneous mutation to rifampin resistance of CGSC1927
JR102	*lac valS*(Ts) *relA*$^+$ *metA rpoB*	+	−	ND	P1 transduction from JR100 into NC51
NC51	*lac valS*(Ts) *relA*$^+$	+	−	+	Derivative of B/rA (14)
NC52	*lac valS*(Ts) *relA*$^-$	+	+	+	Derivative of B/rA (14)
RYH101	*lac valS*(Ts) *relA*$^+$ *nuvC*	−	+	−	Spontaneous mutant of NC51

[a] −, No colony formation; +, colony formation during intermittent near-UV irradiation.
[b] +, tRNA has 4-Srd; −, no 4-Srd in tRNA.
[c] ND, Not determined.

colonies or had remained microcolonies. The intermediate-sized colonies contained mutant, near-UV irradiation-resistant bacteria which had either a shortened growth delay or no growth delay. These mutant colonies were picked, suspended, diluted, and streaked on an agar plate with an inoculating loop. This plate was incubated with intermittent near-UV irradiation at 30°C. After 20 h, several near-UV irradiation-resistant colonies had formed on each streak (except on the control streak of the parent, NC51). One colony of each streak was subjected to another cycle of purification without near-UV irradiation. Colonies on the second purification plate were transferred to a grid plate for temporary storage and further characterization. Since only a few hundred bacteria were originally plated, each mutant colony on the plate must have arisen from an independent mutational event during the initial growth of the microcolonies. Most of the mutations are assumed to be spontaneous, since near-UV irradiation at the fluence used would result in a mutation rate only slightly above background (7).

Screening and classification of near-UV irradiation-resistant mutants. A spot test has been developed to screen the near-UV irradiation-resistant mutants for the Rel phenotype, using NC51 (*relA*$^+$) and NC52 (*relA*) as a model system. Samples (10 μl) of a diluted cell suspension to be tested were placed together with small amounts of the tryptophan analog methyltryptophan on an agar plate. The plate was incubated for 3 days at 30°C. With very low amounts of methyltryptophan (<0.035 μg per 10-μl drop), both NC51 and NC52 formed colonies; with very large amounts (>0.7 μg) neither strain grew. With intermediate amounts, all NC51 cells formed small colonies, whereas none or only a few NC52 cells formed visible colonies. An amount of 0.35 μg per 10-μl drop gave the best distinction between NC51 and NC52 and was therefore chosen to screen the near-UV irradiation-resistant mutants isolated. During incubation of the plate, the methyltryptophan diffuses into the agar and is thereby diluted. Thus, the method consists of subjecting the cells to a temporary amino acid starvation, from which the Rel$^+$ cells recover better than Rel$^-$ cells. (The reason for this differential behavior is not known, but similar amino acid effects have been observed previously [2].) It was later found that this test is not specific for Rel mutants, since certain tRNA modification mutants also did not grow with methyltryptophan (see below).

As a further screen, we spotted dilutions of the near-UV irradiation-resistant strains on glucose minimal medium plates and on glucose minimal medium plates supplemented with thiamine, since early results indicated that many near-UV irradiation-resistant strains were auxotrophic for thiamine.

Classification of near-UV irradiation-resistant mutants. Near-UV irradiation-resistant mutants are those which have either no growth delay (tRNA modification mutants) or a shortened growth delay (Rel mutants; see above). We have isolated 105 independent near-UV irradiation-resistant mutants as described above. The mutants were classified I through IV (Table 2) according to their pattern of growth on four different selective plates (LB medium, glucose minimal medium with methyltryptophan, glucose minimal medium alone, and glucose minimal medium with thiamine; see above). The four classes were tentatively interpreted as follows: class I, growth on all four plates (tRNA modification mutants, not involving thiamine deficiency, and Rel$^+$ [56% of total]); class II, growth on all plates except those with methyl-tryptophan (Rel$^-$ phenotype and a special type of tRNA modification [16% of total]); class III, growth only on LB medium and glucose minimal medium with thiamine (tRNA modification involving

TABLE 2. Classification of near-UV irradiation-resistant mutants (see text)

Class	Colony formation on different growth media[a]			Strains isolated	
	Glucose minimal	Glucose minimal + methyltryptophan −	Glucose minimal + thiamine	No.	% of total
I	+	+	+	59	56
IIa[b]	+	−	+	14	13
IIb				3	3
III	−	−	+	28	27
IV	−	−	−	1	1

[a] +, Colony formation, −, no colony formation.

[b] The distinction between class IIa and IIb was made on the basis of the observed mass increase in the culture after amino acid starvation: classes IIa and IIb behave like Rel$^+$ and Rel$^-$ bacteria, respectively (Fig. 1).

thiamine deficiency and Rel$^+$ [27% of total]); class IV, growth on LB medium only (tRNA modification involving auxotrophy other than thiamine [only 1 of 105]). After a temperature shift to 42°C, this class IV mutant responded like NC51, which means that it had the Rel$^+$ phenotype. By using a standard test for 25 auxotrophic deficiencies, it was found that the mutant could grow very slowly if the medium was supplemented with phenylalanine. It is conceivable that this mutant is a double auxotroph, one deficiency being in phenylalanine synthesis and the other deficiency being a leaky mutation in a gene for a metabolite not present in the set of the 25 test compounds used. It was concluded that this mutant probably also owes its near-UV irradiation resistance to a deficiency in tRNA modification. The phenylalanine requirement may be related to the lack of tRNA modification, since phenyalanyl-tRNA is one of the major 4-Srd-containing tRNAs (14).

Cultures of the class II mutants (i.e., those which include the phenotypic Rel$^-$ strains) were subjected to valine starvation by a temperature shift from 30 to 42°C (all strains had the temperature-sensitive valyl-tRNA synthetase [Table 1]), while mass accumulation (optical density at 460 nm) was followed. Rel$^+$ bacteria show the stringent response, a shutoff of or reduction in rRNA and tRNA syntheses, whereas Rel$^-$ bacteria continue to make rRNA and tRNA. This difference shows up as a difference in mass accumulation (Fig. 1) which permitted the subdivision of class II mutants into subclasses IIa (Rel$^-$, 3%) and IIB (Rel$^+$, 13%).

Lack of 4-Srd in tRNA. One of the mutants from class III, RYH101, was chosen for further study. The fluorescence emission and excitation spectra of tRNA isolated from near-UV irradiated and unirradiated RYH101 were found to be identical to those from unirradiated *E. coli* B/r, i.e., lacking a maximum at 396 nm (12), whereas the emission spectrum of 4-Srd-containing tRNA of irradiated *E. coli* B/r showed a normal maximum at 396 nm (data not shown). Thus, since the spectra from near-UV irradiated tRNA of RYH101 were indistinguishable from those of the unirradiated controls, it is inferred that the tRNA of RYH101 lacks 4-Srd. This was expected because the strain is near-UV resistant without being Rel$^-$.

A more direct proof of lack of 4-Srd is provided by the thionucleoside pattern of a digest of ^{35}S-labeled tRNA from RYH101. Elution of the thionucleosides from a cellulose phosphate column revealed that the pattern of RYH101 was exactly like that of its 4-Srd-containing parent, NC51 (8), except that the large peak of 4-Srd was lacking. All other thionucleosides were present in normal amounts (data not shown).

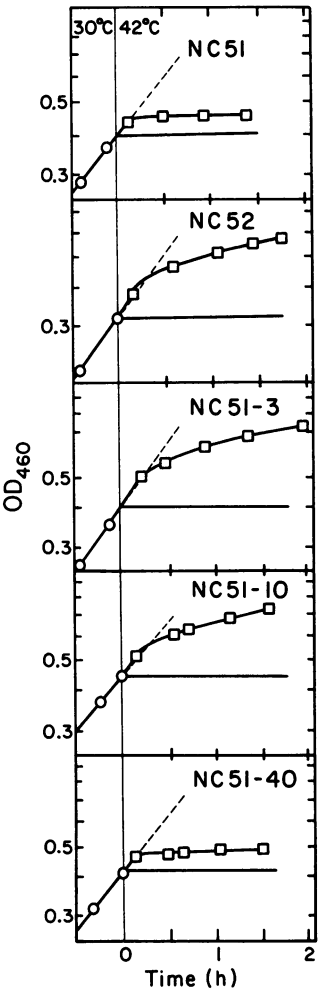

FIG. 1. Growth of Rel mutants during amino acid starvation. Cultures of NC51 (*relA*$^+$), NC52 (*relA*), and three class II mutants isolated during this work (Table 2), NC51-3, NC51-10, and NC51-40, growing in glucose-Casamino Acids medium at 30°C were shifted to 42°C (t = 0). "Relaxed" strains, such as NC52, show a greater increase in mass after amino acid starvation than do "stringent" strains, such as NC51. OD_{460}, Optical density at 460 nm.

Deficiency in one of the thiolating enzymes in vitro. Measurements of the activity of the two enzymes, factors A and C, previously found to be responsible for 4-Srd modification (8) are presented in Table 3. Whereas the extract from RYH101 gave only background activity by itself or when supplemented with factor A, the addition of exogenous factor C produced large amounts of 4-Srd. The extract from RYH101 had no apparent inhibitory effect upon the activity of a mixture of purified factors A and C. Thus, it is

TABLE 3. Activity of the 4-Srd-forming system in RYH101

Additions	4-Srd-forming activity[a] (U/mg of RYH101 protein)
None	1.0
Factor A	2.2
Factor C	14.0
Factors A and C	13.9

[a] Extracts were prepared and assayed as previously described (8). All activities were normalized to 1 mg of extract protein. Factor A was added as 7 U of an enzyme prepared from *E. coli* B, purified through the cellulose phosphate column stage (1), 141 U/mg of protein. The factor C supplement (15 U) was the effluent from the same column, 79 U/mg of protein. Neither supplement showed activity by itself.

concluded that RYH101 is deficient in factor C activity, but has normal factor A activity.

Genetic and physiological characterizations. Strain RYH101 would grow equally well on thiamine, 5-(β-hydroxyethyl)-4-methylthiazole, or thiazole (each 4 μg/ml), suggesting that the mutation lay in an unknown gene involved in the biosynthesis of thiazole. The strain was found to revert to Thi^+. Of 30 such revertants, 26 (or 87%) had also regained near-UV irradiation sensitivity, indicating that the same defect which resulted in the loss of ability to synthesize thiazole also abolished the ability to produce 4-Srd in tRNA. Since the addition of thiazole or thiamine to the medium restored growth but did not produce near-UV irradiation sensitivity, it appears that the lack of thiazole or thiamine is not responsible for the lack of tRNA modification.

Next, the position of the lesion on the *E. coli* chromosomal map was investigated. The known genes for thiamine biosynthesis, *thiA*, *thiB*, and *thiC*, map between *metA* and *rpoB*, at 89.7 min on the *E. coli* chromosome (3). When the $metA^+$ and $rpoB^+$ markers from RYH101 were transduced with phage P1 into JR102 (*metA rpoB*; Table 1), all cotransductants tested (six of six) were Thi^+ and near-UV irradiation sensitive, indicating that the mutation which made RYH101 Thi^- did not map in the region of the cluster of known *thi* genes. However, products of these genes catalyze the conversion of thiazole into thiamine, whereas the fact that RYH101 could grow on thiazole pointed to a defect in an enzyme involved earlier in the pathway, in thiazole synthesis. No genes associated with this earlier pathway were known.

As noted above, the in vitro modification of uridine in tRNA to 4-Srd requires two protein factors, A and C. Factor A maps at *nuvA* (9 min), and the gene for factor C, designated *nuvC*, is roughly located near *his* at 44 min (8). A phage P1 lysate grown on RYH101 was used to transduce histidine prototrophy into a *his* strain (CSH57; Table 1). Of 85 his^+ transductants tested, 14 (16%) were Thi^-. Since the maximum DNA segment that can be transduced with phage P1 corresponds to 2.1 map units (4), it is concluded that the thiazole defect maps between 42 and 46 min on the chromosome, which is consistent with the previous estimate of the *nuvC* position (8).

DISCUSSION

Two enzymes were previously found to be responsible for the 4-Srd modification of tRNA; these were named factors A and C (8). One of the near-UV irradiation-resistant mutants isolated by Ramabhadran (11) was found to lack factor A activity; the gene responsible for this deficiency was named *nuvA* and located at 9 min on the *E. coli* chromosome map (8). A mutant strain deficient in factor C activity had not been previously found; however, when cell extracts from partial diploid strains containing various segments of the *E. coli* chromosome on an F′ plasmid were studied, it was found that strains containing an extra copy of the *his* region of the *E. coli* chromosome had factor C activity that was approximately twofold increased (8). From such observations, the existence of a structural gene for factor C, *nuvC*, mapping near *his* (at 44 min), was postulated. The results obtained here indicate that RYH101 is a *nuvC* mutant; the mutation maps near *his* and simultaneously produces a deficiency in the 4-Srd modification of tRNA and in the activity of factor C required for this tRNA modification. That *nuvC* is also involved in thiazole biosynthesis was unexpected, although a second function for the factor C enzyme has previously been postulated, since it was observed that yeasts, which have no 4-Srd modification in their tRNA, nevertheless contain an enzyme which can substitute for *E. coli* factor C in an in vitro thiolation system (8).

In the presence of thiamine, neither thiazole deficiency nor 4-Srd-deficient tRNA seemed to have any ill effect on the growth of the bacteria. A few Thi^+ revertants from RYH101 (4 of 30) were still near-UV irradiation resistant. We have not mapped these phenotypic revertants; they indicate either intragenic or extragenic suppression. The first case would suggest separate functional regions in the factor C polypeptide.

The exact map position of these mutations remains to be established, but with the availability of a large number of such mutants and with relatively easy screening procedures, the location should be forthcoming. These mutants may also allow further study of the thiazole pathway, which is not known. It is particularly intriguing to speculate whether factor C, which transfers

sulfur from cysteine to tRNA uridine to form 4-Srd (10), may also transfer sulfur (or move the whole cysteine moiety) to another acceptor to form thiazole.

We have not further studied any of the other (Rel^+ Thi^+) near-UV irradiation-resistant mutants obtained in this work. Whether these other mutants are all *nuvA* or whether additional gene functions are involved in the phenomenon of near-UV irradiation resistance must be established in the future.

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