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Specific Action of 4-Nitropyridine 1-Oxide on *Escherichia coli* K-12 Pro$^+$ Strains Leading to the Isolation of Proline-Requiring Mutants: Isolation and Characterization of Pro$^-$ Mutants

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A specific action of 4-nitropyridine 1-oxide on *Escherichia coli* K-12 Pro$^+$ strains leading to highly efficient, selective isolation of Pro$^-$ mutants is described. Incubation of Pro$^+$ cells with a sublethal concentration of 4-nitropyridine 1-oxide in Penassay broth gave Pro$^-$ mutants, which lacked either the biosynthetic pathway of proline from glutamic acid to glutamyl γ-phosphate (*proB*$^-$) or the pathway from glutamyl γ-phosphate to glutamic γ-semialdehyde (*proA*$^-$) or both. Pro$^-$ mutants, which have the metabolic block between Δ^1 pyrroline-5-carboxylate (the cyclized dehydration product of glutamic γ-semialdehyde) and proline (*proC*$^-$) were not found among survivors. Treatment of Pro$^+$ cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine led to isolation of all three types of Pro$^-$ mutants, suggesting that the action of 4-nitropyridine 1-oxide on Pro$^+$ cells is apparently distinct from the action of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. F-duction and interrupted mating experiments led to determination of the correlation between proline loci and the biosynthetic pathway of proline from glutamic acid.

4-Nitroquinoline 1-oxide has been known to show characteristic biological activities (14), similar to those revealed by acridine dyes (23), such as bactericidal action (2), prophage induction from lysogenic bacteria (13), mutagenic actions toward bacteria (28), and carcinogenicity (26). On the other hand, 4-nitropyridine 1-oxide (4NPO), a pyridine analogue of 4-nitroquinoline 1-oxide, has been known to exhibit little biological activity (14); for instance, the compound is inactive for induction of prophage (13) and shows only very weak carcinogenicity (3).

During the course of a series of investigations on the structure and biological activities of compounds with pyridine, quinoline and acridine skeletons, we found that 4NPO can effectively act on *Escherichia coli* K-12 Pro$^+$ strains, leading to the isolation of proline-requiring (Pro$^-$) mutants in large numbers. We found that Pro$^-$ mutants isolated by this method have a metabolic block between glutamic acid (Glu) and glutamic γ-semialdehyde (GSA) in the biosynthetic pathway from Glu to proline and may be characterized as *proA*$^-$ and/or *proB*$^-$. The major mechanism of the action of 4NPO was shown to be selection; i.e., 4NPO is more toxic for *pro*$^+$ cells than for *proA*$^-$ and *proB*$^-$ (but not for *proC*$^-$) cells. This paper describes the standard method of isolation of Pro$^-$ mutants by treatment of *Escherichia coli* K-12 Pro$^+$ cells with 4NPO and the genetic and biochemical characterizations of Pro$^-$ mutants isolated. Further evidence on the metabolism of 4NPO by Pro$^+$ or Pro$^-$ cells and mechanism of selective specificity of the agent toward Pro$^+$ and Pro$^-$ cells reported in the accompanying paper (19). A preliminary report has appeared (17).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this work were *E. coli* K-12 and *Salmonella typhimurium* LT-2 derivatives (Table 1). Pro$^-$ mutants other than those isolated by treatment with 4NPO were obtained by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) or by ultraviolet irradiation. W strains of *E. coli* and JM strains of *S. typhimurium* were supplied by Y. Hirota, Osaka University, and T. Miyake, Keio University, respectively.

Materials and media. 4NPO (27), NTG (24), and Δ^1-pyrroline 5-carboxylic acid (PC) (34) were synthe-

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TABLE 1. *Bacterial strains used*

Strain	Relevant genotype[a]	Origin or source
E. coli K-12		
W3110	F⁻	
W3630	F⁻ *mal-5*⁻	
W3747	F⁺13 (F-*lac*⁺ *proC*⁺ *ade*⁺) *met*⁻	
W4573	F⁻ *ara-2*⁻ *lac-85*⁻ *gal-2*⁻ *mal-1*⁻ *mtl*⁻ *xyl-2*⁻ *str*ʳ	
JE177	F⁻ R⁺$_{100-1}$ (*str*ʳ *cml*ʳ *tet*ʳ *sul*ʳ) *mal-5*⁻	W3630
JE1031	HfrH *met*⁻ *thi*⁻	
JE2133[b]	F⁻ *ara-2*⁻ *proA1*⁻ *lac85*⁻ *gal-2*⁻ *mal-1*⁻ *mtl*⁻ *xyl-2*⁻ *str*ʳ	W4573
JE2265[c]	F⁻ *ara-2*⁻ *tonB proA1*⁻ *tsx lac-85*⁻ *gal-2*⁻ *mal-1*⁻ *mtl*⁻ *xyl-2*⁻ *str*ʳ	JE2133
KE113[b]	F⁻ *ara-2*⁻ *proB3*⁻ *lac-85*⁻ *gal-2*⁻ *mal-1*⁻ *mtl*⁻ *xyl-2*⁻ *str*ʳ	W4573
KE114[d]	F⁻ *ara-2*⁻ *lac-85*⁻ *proC36*⁻ *gal-2*⁻ *mal-1*⁻ *mtl*⁻ *xyl-2*⁻ *str*ʳ	W4573
KE125[d]	F⁻ *ara-2*⁻ *proB17*⁻ *lac-85*⁻ *gal-2*⁻ *mal-1*⁻ *mtl*⁻ *xyl-2*⁻ *str*ʳ	W4573
KE126[d]	F⁻ *ara-2*⁻ *proA18*⁻ *lac-85*⁻ *gal-2*⁻ *mal-1*⁻ *mtl*⁻ *xyl-2*⁻ *str*ʳ	W4573
χ646	F'ORF-206 (F-*lac*⁺ *proB*⁺ *proA*⁺) *lacy*⁻	(11)
S. typhimurium LT-2		
JM108[e]	*proA*⁻	pro-27
JM109[e]	*proC*⁻	pro-150

[a] Genetic symbols are those used by Taylor and Trotter (31).

[b] Isolated after 4NPO treatment of W4573 and characterized as *pro*⁻.

[c] Derived from JE2133 by spontaneous mutation to resistance to phage T_1 and T_6.

[d] Isolated after treatment with NTG followed by penicillin screening and characterized as *pro*⁻.

[e] Kindly supplied by T. Miyake (25).

sized in our laboratory according to the literature. Dihydrostreptomycin sulfate (SM) was purchased from Takeda Chemical Ind., Osaka.

Penassay broth (Difco) was used for cultivation of microorganisms, 4NPO treatment, and conjugation experiments. Synthetic Davis glucose medium (12) was used for penicillin screening techniques. A complete EMB-glucose-agar medium was used for scoring survivors after 4NPO treatment. Synthetic Davis glucose-agar medium and EM-glucose-agar medium (20) were used for characterization of auxotrophic mutants. Synthetic Davis sugar-agar media (Davis-arabinose, Davis-lactose, and Davis-galactose) with necessary supplements were used for characterizing sugar fermentation and for selection of prototrophic recombinant colonies. Glucose salt mineral medium (34) was used for the accumulation of biosynthetic intermediates from glutamic acid to proline. Antibiotics were added as indicated. Tris(hydroxymethyl)aminomethane-malate (TM) buffer (1) was used for NTG treatment. The pH of media was 7.0 unless otherwise noted.

Method for selective isolation of Pro⁻ mutants by treatment of Pro⁺ strain with 4NPO. A clone of Pro⁺ strain was inoculated into 5 ml of Penassay broth and incubated at 37°C overnight, at which time the titer of the culture had reached about 5×10^8 cells per ml. The culture was diluted 10^{-3}, and 0.2 ml of the diluted culture was added to 100 ml of Penassay broth together with 0.1 to 0.5 ml of 0.1% aqueous 4NPO. After the mixed culture was incubated on a reciprocal shaker at 37°C for 20 to 26 h, 0.1 ml of the culture was plated after appropriate dilutions on EMB-glucose-agar medium. Colonies appearing on this medium were replica-plated onto Davis glucose-agar with or without 40 μg of L-proline per ml.

Isolation of Pro⁻ mutants by NTG treatment of Pro⁺ strain. NTG treatment and penicillin screening methods were carried out by the procedures of Adelberg et al. (1) and Lederberg and Zinder (21). Pro⁺ cells harvested from exponential-growth-phase culture (ca. 10^8 cells per ml) were suspended in TM buffer (pH 7.0) containing 300 μg of NTG per ml, and the mixture was incubated at 37°C for 30 min. Cells were harvested and suspended in Penassay broth (ca. 10^7 cells per ml), and the culture was incubated at 37°C overnight. Cells were collected by centrifugation and treated with 300 units of penicillin G potassium (Takeda Chemical Ind., Osaka, Japan) per ml in Davis-glucose at 37°C for 6 h. Pro⁻ mutants formed were characterized by replica plating on Davis glucose-agar supplemented with or without 40 μg of L-proline per ml. Auxotrophic mutants other than *pro*⁻ were characterized similarly.

Cross-feeding test. Cross-feeding testing was performed for syntrophism between *pro*⁻ mutants of *E. coli* and *proA* and *proC* mutants of *S. typhimurium* LT2, which possess metabolic blocks in the pathway of proline biosynthesis, i.e., JM108 *proA*⁻, which is unable to synthesize GSA (or its cyclized form, PC), and JM109 *proC*⁻, which lacks the reducing enzyme for PC.

A streak across a Davis glucose-agar plate was made with a loopful of Pro⁻ culture to be tested. Perpendicular to this streak, a loopful culture of JM108 or JM109 was spread so that the streaks were separated at one end by about 3 mm. After incubation at 37°C for 48 h, areas of growth of cells around the cross of the two streaks were checked. By this test, *proA*⁻ and *proB*⁻ could be distinguished from *proC*⁻.

Assay for accumulation of biosynthetic intermediates of proline in Pro⁻ mutants. Accumulation of PC in *proC*⁻ cells was assayed by the method of Strecker (29) and Tristram and Thurston (33). To distinguish *proA*⁻ from *proB*⁻, accumulation of glutamohydroxamic acid was assayed by the method of Grossowicz et al. (15).

Infection with and elimination of F factors. The F13 (F *lac*⁺ *proC*⁺ *ade*⁺) and F'ORF-206 (F *lac*⁺ *proB*⁺

proA$^+$) factors were transferred from appropriate F′ (donor) strains into F$^-$ (recipient) strains by mixing equal volumes of exponential-phase cultures of F′ (ca. 4×10^8 cells/ml) and F$^-$ (ca. 2×10^7 cells/ml) cultures, respectively, and the mixed culture was incubated at 37°C for 1 h. Elimination of F factors from male cells was carried out with acridine orange (16) or sodium dodecyl sulfate (32).

P1 transduction. The method of transduction described by Lenox (22) was generally followed. Recipient bacteria were grown in L broth to a concentration of 2×10^8 to 3×10^8 cells per ml. The culture was chilled and centrifuged, and the cells were harvested and suspended in 1/50 volume of L broth. The cell suspension was mixed with a few milliliters of P1 *vir* lysate containing 2.5×10^{-3} M $CaCl_2$ to give a multiplicity of infection of 0.1 to 0.3. This adsorption mixture was kept at 37°C for 20 min and then centrifuged. The pellet was washed with synthetic Davis medium without glucose and resuspended in the same medium, and portions were spread on suitable plates for selection of transductants.

Mating procedures. A 1-ml portion of JE1031 (Hfr-H) culture and 10 ml of Pro$^-$ F$^-$ recipient culture derived from W4573 (*str*^{r}) strain (cell titer of each exponential-phase culture, 2×10^8 to 3×10^8 cells per ml) were mixed and incubated without shaking at 37°C for 5 min. A 0.5-ml portion of the mixed culture was diluted without shearing into 50 ml of fresh Penassay broth prewarmed at 37°C, and the diluent was further incubated at the same temperature. Samples of 2 ml were removed from the mating mixture at certain intervals and transferred to sterilized tubes. The samples were violently agitated on an S-5 Mixer (Taiyo Corp.) for 30 s to separate mating partners. Samples were then immediately withdrawn for plating on selective media using soft agar plus SM at 100 μg/ml. Selection of *pro*$^+$*str*^{r}, *lac*$^+$*str*^{r}, and *gal*$^+$*str*^{r} recombinants were made on Davis glucose-agar plus SM, Davis lactose plus SM and L-proline, and Davis galactose plus SM and L-proline, respectively.

RESULTS

Selective isolation of proline-requiring mutants of *E. coli* K-12 by treatment with 4NPO. W4573 Pro$^+$ strain (inoculum size, 10^3 cells per ml) was incubated with 4NPO at various concentrations in Penassay broth at 37°C for 24 h. Survivors were analyzed for the proline growth requirement. Representative results are shown in Table 2. It was found that proline-requiring (Pro$^-$) mutants were isolated among survivors in very large numbers after treatment with 4NPO at sublethal concentrations, i.e., 2 or 3 μg/ml. Pro$^-$ cells thus obtained were stable after many generations, and spontaneous reverse mutation rates of those Pro$^-$ mutants to Pro$^+$ were 10^{-8} to 10^{-10}. Formation of auxotrophic mutants other than Pro$^-$ was not observed.

4NPO was found to act on *E. coli* K-12 and three derivatives, W3630, W3110 and JE177 (R100-1$^+$), although the frequency of Pro$^-$ mutants formed varied from strain to strain (Table 3). In every case, however, the isolation frequency of Pro$^-$ cells was maximum when Pro$^+$ cells were treated with sublethal concentrations of 4NPO. In further experiments, strain W4573 was mainly used.

TABLE 2. *Elective isolation of pro$^-$ mutants of E. coli K-12 W4573*

Expt no.	Inoculum size (cells/ml)	4NPO concn (μg/ml)	Viable counts (cells/ml)	No. of *pro*$^-$/no. of colonies tested	% *pro*$^-$
1	1.1×10^3	0	6.6×10^9	0/186	0
		1.0	6.7×10^9	0/256	0
		2.0	1.8×10^6	41/149	27.5
		3.0	1.4×10^4	102/130	78.5
2	1.8×10^3	0	4.6×10^9	0/193	0
		1.0	5.4×10^9	0/242	0
		2.0	2.5×10^4	201/231	87.0
		3.0	1.1×10^2	0/10	0
3	2.9×10^3	0	6.7×10^9	0/269	0
		1.0	6.0×10^9	0/234	0
		2.0	6.2×10^5	395/469	84.2
		3.0	1.3×10^3	34/113	30.1

Effects of inoculum size of Pro$^+$ cells, nutrient conditions of the medium, and incubation temperature were checked. Experiments with W4573 (Pro$^+$) cells showed that when 4NPO at 2.0 μg/ml in Penassay broth was used, apposite inoculum size of Pro$^+$ cells was 10^3 to 10^4 cells per ml. It was not possible to obtain Pro$^-$ mutants when the nutrient medium was substituted with a synthetic Davis glucose medium supplemented with 40 μg of L-proline per ml. With respect to incubation temperature, an optimum temperature for the 4NPO treatment was 37°C; at 4°C, formation of Pro$^-$ mutants was not observed. The results suggested that growth of the cells is essential for the action of 4NPO. It was further found that treatment of W3110 Pro$^+$ cells with ultraviolet irradiation or NTG prior to the addition of 4NPO increased the frequency of Pro$^-$ formation 3.8- or 6.9-fold, respectively.

Kinetics of the 4NPO treatment. An overnight culture (ca. 5×10^8 cells/ml) of W4573 Pro$^+$ was appropriately diluted in Penassay broth with or without 2.0 μg of 4NPO per ml to give cell concentrations of 10^4 cells/ml. The cultures were incubated with shaking at 37°C, and the survivors were analyzed at intervals. Representative results are shown in Fig. 1. In 4NPO broth, the titer of Pro$^+$ cells, after a lag of 2 h, rapidly decreased to 10^{-2} in 6 h. Then, the cell titer of the culture began to increase exponentially, when a substantial fraction of survivors

TABLE 3. *Effect of strains on the isolation of pro⁻ mutants by 4NPO treatment*

E. coli strain	4NPO concn (μg/ml)	% pro⁻[a]							
W4573	2.0	59.9	0	0	27.5	69.4	87.0	84.2	0
	3.0	0	0	0	78.5	–	0	30.1	–
K-12	3.0	73.4	17.7	0	2.3	0	0		
	4.0	50.0	47.9	30.5	–	14.0	0		
	5.0	71.6	0	0	0	–	0		
	6.0	7.5							
W3630	1.0	18.5	8.9	38.5	0.9	17.9			
	2.0	59.3	53.2	–	100	–			
	3.0	–	0	0	93.9	1.7			
W3110	3.0	0	0	0	0	0	0		
	4.0	0	0	32.8	0	0	0		
JE177	1.0	42.9	0						
	1.5	34.5	–						
	2.0	57.8	100						
	2.5	99.5	100						
	3.0	–	11.6						

[a] Two to eight experiments were carried out using each strain. Each culture contained about 10^4 to 10^8 cells per ml; the number of colonies tested in each experiment was about 200. Results are expressed as (number of *pro* mutants)/(number of colonies tested) × 100.

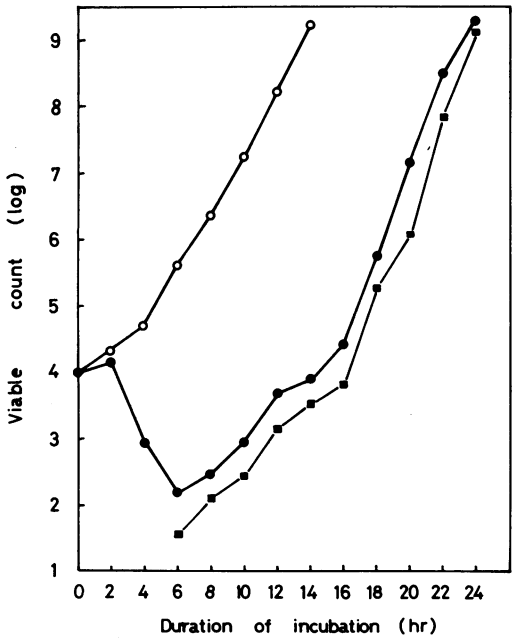

FIG. 1. *Kinetics of 4NPO treatment of W4573 pro⁺. An overnight culture (ca. 5 × 10^8 cells/ml) of W4573 pro⁺ was appropriately diluted in Penassay broth with or without 2.0 μg of 4NPO per ml to give cell concentrations of 10^4 cells/ml. The cultures were incubated with shaking at 37°C, and plating with suitable dilutions at intervals was on EMB-glucose-agar. Colonies were replica plated onto Davis glucose-agar with or without L-proline (40 μg/ml) to examine the frequency of pro⁻ cells. Symbols: ○, viable count without 4NPO; ●, viable count with 4NPO; ■, viable count of pro⁻ cells formed in the culture with 4NPO.*

was found to be Pro⁻ cells. Frequencies of Pro⁻ cells after 2, 6, 12, and 18 h of incubation were 0, 29, 31.3, and 31.7%, respectively. In broth without 4NPO, Pro⁺ cells began to grow exponentially immediately after incubation started; Pro⁻ cells were not isolated among survivors.

Biochemical characterization of Pro⁻ mutants. It has been reported that in enteric bacteria, proline is synthesized from glutamic acid via the following pathways, governed by three *pro* genes, *proA*, *proB*, and *proC* (4, 5, 6, 29, 30, 35): Glu → GP → GSA → PC → proline.

To determine which step of proline biosynthesis is blocked in proline-requiring mutants isolated after the 4NPO treatment, the growth response of 58 Pro⁻ clones obtained independently by 4NPO treatment of W4573 Pro⁺ to Glu, PC, proline, hydroxyproline, ornithine, arginine, and α-ketoglutaric acid in Davis glucose medium was first tested. All Pro⁻ mutants tested could grow in the presence of either PC or proline but not by other supplements. The result indicated that those Pro⁻ mutants have a metabolic block(s) between Glu and GSA.

Second, further characterization of Pro⁻ mutants as either those having metabolic block between Glu and Gp or GP and GSA was carried out by examining possible accumulation of GP identified as γ-glutamohydroxamic acid.

The Pro⁻ mutants were classified into two types, i.e., those that are capable of synthesizing GP and those that are not (Fig. 2a).

Examination of possible accumulation of PC in Pro⁻ mutants was also carried out (Fig. 2b). As expected, accumulation of PC was not observed in ten Pro⁻ clones tested as in W4573 Pro⁺.

Thirdly, the syntrophism test of 58 Pro⁻ mutants and authentic *proA* and *proC* strains of *S. typhimurium*, JM108 and JM109, was carried out. All the mutants were cross-fed not by JM109 *proA*⁻ but by JM109 *proC*⁻. The result further supported the previous observation that the Pro⁻ mutants tested cannot synthesize GSA or PC and may be genetically *proA* or *proB*.

The results, together with those obtained with Pro⁻ mutants isolated after treatment of W4573 *Pro*⁺ cells with NTG, are summarized in Table 4. A remarkable difference existed between the results obtained by treatment with 4NPO and NTG; whereas the NTG treatment led to isolation of all three types of *pro*⁻ mutants, *A*, *B*, and *C*, the treatment with 4NPO specifically afforded either *proA* and/or *proB* but never *proC*.

Genetic characterization of Pro⁻ mutants. As discussed above, three *pro* genes, *A*, *B*, and *C*, have been genetically identified with respect to proline biosynthesis in *E. coli* (7-10, 31). Moreover, it has been suggested that *proA* and *proB* genes correspond to biosynthetic pathways prior to GSA, and that *proC* is the structural gene for PC reductase. Chromosomal

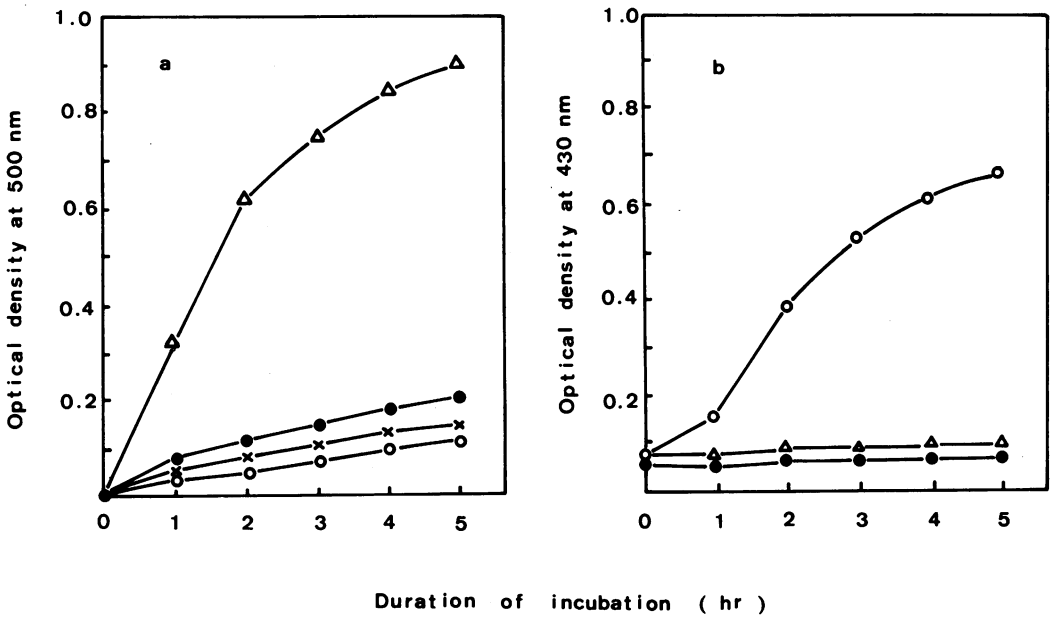

FIG. 2. *Accumulation of (a) GP and (b) PC. For procedure, see Materials and Methods. (a) Incubation was initiated by the addition of L-Glu and hydroxylamine into Pro⁻ or Pro⁺ cell suspensions. Symbols: ●, W4573 pro⁺; △, JE2133 pro⁻ (4NPO); ×, KE113 pro⁻ (4NPO); ○, KE114 pro⁻ (NTG). (b) Incubation was initiated by the addition of L-Glu into Pro⁻ and Pro⁺ cells suspensions. Symnbols as in (a).*

TABLE 4. *Classification of pro⁻ mutants by biochemical analysis*

Lacking steps in proline biosynthesis	No. of *pro*⁻ (4NPO treatment)		No. of *pro*⁻ (NTG treatment)	
	By syntrophism	By accumulation of intermediates	By syntrophism	By accumulation of intermediates
Glu → GP	58 (100%)	2 (20%)	27 (84%)	2 (25%)
GP → GSA		8 (80%)		5 (63%)
PC → proline	0 (0%)	0 (0%)	5 (16%)	1 (12%)
Total no. of clones examined	58	10	32	8

mapping of *pro* genes of proline-requiring mutants obtained by 4NPO treatment was then carried out to correlate without ambiguity proline loci and biosynthetic processes for proline in *E. coli*.

(i) F-duction of *pro* gene. For F-duction experiments, strains W3747 harboring F13 (F *lac*$^+$ *proC*$^+$ *ade*$^+$) (18) and χ646 harboring F'ORF-206 (F *lac*$^+$ *proB*$^+$ *proA*$^+$) (11) were used as F' strains. Cultures (ca. 2×10^8 cells per ml) of W3747 (F13$^+$) and Pro$^-$ Lac$^-$ mutants in the exponential phase were mixed in a ratio of 10:1. After incubation at 37°C for 1 h, the mixture was plated on EMB-lactose-agar plus 100 μg of SM per ml, where only recipient bacteria could grow. Lac$^+$Str^r colonies grown on the plate were picked at random from EMB-lactose-agar and replica plated on Davis glucose-agar. When the Pro$^-$ mutant KE114 obtained by the NTG treatment and characterized as having the metabolic block between PC and proline was used as recipient, all Lac$^+$Str^r F-ductants formed were Pro$^+$. When the Pro$^-$ mutant JE2133 or KE113 obtained by the 4NPO treatment and characterized as having the block between GP and GSA or Glu and GP, respectively, was used as recipient, all Lac$^+$Str^r F-ductants were Pro$^-$. F-duction experiments with χ646 (F *lac*$^+$ *proB*$^+$ *proA*$^+$) as F' strain further indicated that Pro$^+$ F-ductants were formed by the cross of χ646 with Pro$^-$ mutants JE2133, KE113, and KE125 (which possess the same metabolic block as JE2133), as recipient but not with KE114. The result indicated that mutation sites of Pro$^-$ mutants isolated after 4NPO treatment are not located on F13 but are on F'ORF-206.

(ii) Interrupted-mating experiments. Preliminary experiments of the cross of JE1031 (HfrH) as donor and JE2265 (*pro*$^-$ *ara-2*$^-$ *tonB tsx lac-85*$^-$ *gal-2*$^-$ *str*^{r}), which was obtained by 4NPO treatment and has the metabolic block between GP and GSA, suggested that the location of the *pro* gene is between *tonB* and *lac*. Interrupted-mating experiments were carried out to determine the chromosomal location of *pro* (Glu → GP) and *pro* (Gp → PCA) genes. Figure 3 shows the kinetics of recombinant formation of interrupted mating of JE1031 (HfrH *pro* (Glu → GP) and *pro* (GP → PC) genes. GP] *lac-85*$^-$ *gal-2*$^-$ *str*^{r}). Average interlocus distance between *pro* (Glu → GP) and *lac-85* was 3.4 min, and the distance between *pro* and *gal-2* was 6.3 min. In the same manner, interrupted mating of JE1031 HfrH and JE2133 *pro*$^-$ (GP → GSA) was carried out. The result indicated that the average distance between *pro* (GP → GSA) and *lac-85* was 5.3 min. This evidence indicates that the interlocus distance between *pro* (GP → GSA) and *pro* (Glu → GP)

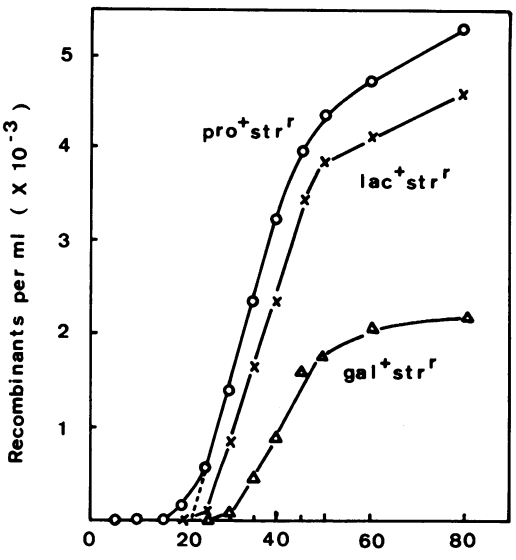

FIG. 3. *Kinetics of recombinant formation in an interrupted-mating cross of JE1031 HfrH and KE113 pro*$^-$ *(Glu → GP). Cultures in the exponential growth phase of JE1031 and KE113 were mixed at a ratio of 1:10 and incubated without shaking at 37°C for 5 min. Mixed cultures were diluted 10^{-2} without shearing into fresh Penassay broth prewarmed at 37°C, and the diluent was further incubated. Samples removed from the mating mixture at certain intervals were violently agitated on a mixer for 30 s. After suitable dilutions, pro*$^+$ *str*^{r}, *lac*$^+$*str*^{r}, *and gal*$^+$*str*^{r} *recombinants were selected on Davis glucose-agar plus SM at 100 μg/ml, Davis lactose-agar plus SM and* L*-proline, and Davis galactose-agar plus SM and* L*-proline, respectively.*

is about 1.9 min, and the gene order of loci involving three *pro* is *ara*, *tonB*, *pro* (GP → GSA), *pro* (Glu → GP), *lac*, *pro* (PC → proline), *tsx*, and *gal*. It is concluded that *pro* (GP → GSA) and *pro* (Glu → GP) correspond to *proA* and *proB*, respectively, in the genetic map of *E. coli* by Taylor and Trotter (31). Transduction experiments with phage P1 *vir* further revealed a close linkage between *proA* and *proB* loci the number of transductants between *proA*$^-$ and *proB*$^-$ were consistently smaller (one-fifth to one-tenth) than those of transductants between *proA* or *proB* and *proC* or with wild type (data not shown). Frequencies of cotransduction between *proA1* or *proB3* or *lac-85* mutations in JE2133 or KE113 were shown to be less than 0.21 and 0.40%, respectively.

DISCUSSION

The results showed that 4NPO, which is known to exhibit little biological activity, can act on *E. coli* K-12 Pro$^+$ strains, leading to

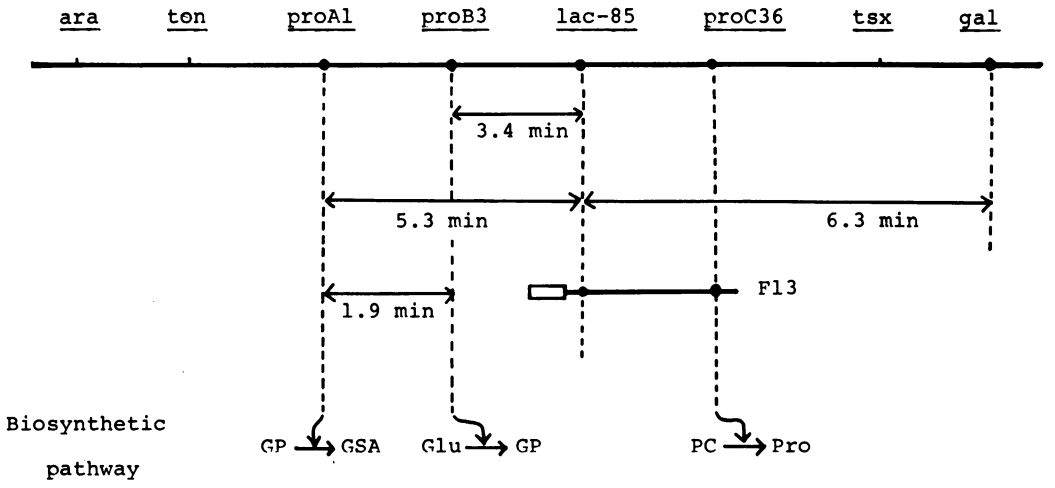

FIG. 4. *Correlation between loci and processes of the biosynthetic pathway of proline. Results are from the interrupted-matings crosses of JE1031 HfrH × KE113 F⁻ proB⁻ (Glu → GP) and JE1031 × JE2133 F⁻ proA⁻ (GP → GSA) and from F-duction of proC gene from W3747 F13⁺ to KE114 proC⁻ (PC → proline).*

highly efficient, selective isolation of Pro⁻ mutants. It was also shown that isolation of Pro⁻ mutants was highest when a sublethal concentration of 4NPO in Penassay broth was used (Tables 2 and 3). Kinetic studies of the 4NPO treatment of W4573 Pro⁺ (Fig. 1) showed that after a short lag time, the titer of Pro⁺ cells quickly decreased 10^{-2} to 10^{-3}. Then the cell titer began to again increase exponentially; Pro⁻ mutants appeared among survivors, and their cell titer increased thereafter. The results suggest that the agent might have a selective affinity toward Pro⁺ and Pro⁻ cells; i.e., the agent is more toxic for Pro⁺ than for Pro⁻.

Biochemical characterization of Pro⁻ mutants thus formed indicated that all the Pro⁻ mutants lack the biosynthetic pathway of proline from Glu to GP and/or from GP to GSA. Pro⁻ mutants that have the metabolic block between PC and Pro were not found among the survivors. On the other hand, Pro⁻ mutants obtained by treatment with NTG could be classified into all three types of mutants (Fig. 2 and Table 4). This observation indicates that the specific action of 4NPO on Pro⁺ cells is apparently distinct from the action of NTG.

Three genes, *proA*, *proB*, and *proC*, have been mapped close to *lac* on the chromosome of *E. coli* (7-10, 31). However, the correlation of *proA* and *proB* with the biosynthetic pathway of proline has not been completely settled. Results of F-duction and interrupted-mating experiments (Fig. 3 and 4) led us to the following conclusions. Pro⁻ mutants obtained after 4NPO treatment are *proA* (or *pro-1*) and/or *proB* (or *pro-2*) (but not *proC*) by the system of Taylor and Trotter (31) or of Curtiss (10). *proA*⁻ and *proB*⁻ genes have the metabolic block between GP and GSA and between Glu and GP, respectively. The correlation of three *pro* genes and their functions in the biosynthesis of proline from glutamic acid can therefore be summarized as $\text{Glu} \xrightarrow{proB} \text{GP} \xrightarrow{proA} \text{GSA} \xrightarrow[\text{dehydration}]{\text{nonenzymatic}} \text{PC} \xrightarrow{proC} \text{proline}$.

The data in Table 3 suggested that there was some strain effect on the efficiency of the action of 4NPO. It was further observed that the frequency of Pro⁻ cells isolated after 4NPO treatment fluctuated with every strain used. Pretreatment of bacteria with UV light or NTG prior to treatment with 4NPO apparently increased the frequency of formation of Pro⁻ mutants. These results, together with those of kinetic experiments, lead us to suggest that the mechanism of the specific action of 4NPO may be the selection of *proA*⁻ and *proB*⁻ cells that arose spontaneously in the Pro⁺ culture. Further results on the metabolism of 4NPO and possible mechanisms of the specific action of 4NPO are dealt with in the accompanying paper (19).

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