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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: Mechanism of Action of 4-Nitropyridine 1-Oxide

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Possible mechanisms involved in the action of 4-nitropyridine 1-oxide (4NPO) on *Escherichia coli* K-12 *pro*$^+$ cells in Penassay broth leading to the selective isolation of *proA*$^-$ and/or *proB*$^-$ mutants but not *proC*$^-$ mutant were studied. Reconstruction experiments between *pro*$^+$ and *pro*$^-$ cells, together with experiments on the bactericidal action of 4NPO on *pro*$^+$ and *pro*$^-$ cells, indicated that 4NPO is more toxic for *pro*$^+$ and *proC*$^-$ cells than for *proA*$^-$ and *proB*$^-$ cells. These results, coupled with data indicating little mutagenicity of 4NPO on *E. coli* cells, led us to conclude that the selection of *proA*$^-$ and/or *proB*$^-$ cells that arose spontaneously in the *pro*$^+$ culture is a possible mechanism for the action of 4NPO. Examination of 4NPO sensitivity of *pro*$^+$ transductants derived from *proA*$^-$ and *proB*$^-$ cells with P1 *vir* phage and *pro*$^+$ cells as donor and of *pro*$^+$ spontaneous revertants derived from those *pro*$^-$ cells suggested that 4NPO-sensitive gene(s) should be on, or very close to, the *proA* and *proB* loci and that both products of *proA* and *proB* genes may be involved in the sensitivity of bacteria to 4NPO. The fact that the 4NPO-sensitive allele is dominant over the 4NPO-resistant allele further indicated the possible correlation between gene products of *proA* and *proB* and the 4NPO sensitivity of bacteria. Experiments on metabolic conversion of 4NPO with bacterial cells proved that the major metabolic pathway of the agent is reduction to (possibly via 4-nitroso-) 4-hydroxylamino- and 4-amino-pyridine 1-oxides, and then to 4-aminopyridine. Investigation of the effect of structural modification of 4NPO on the elective selection of Pro$^-$ mutants in Pro$^+$ culture further suggested that the structural feature indispensable for the action of the agent is the hydroxyl-amino or its more oxidized state at the 4 position and the *N*-oxide moiety at the 1 position on the pyridine skeleton. Action of 4NPO in minimal medium was found to be bacteriostatic on *pro*$^+$ cells but not on *pro*$^-$ cells, leading to the formation of long nonseptate multinucleate filament cells on *pro*$^+$ cells. Possible biochemical mechanisms of the selective toxicity of 4NPO for *pro*$^+$ and *pro*$^-$ cells are discussed.

We have found that 4-nitropyridine 1-oxide (4NPO) can act on *Escherichia coli* K-12 Pro$^+$ strains, leading to highly efficient isolation of proline-requiring (Pro$^-$) mutants (7, 8). All Pro$^-$ mutants thus isolated have been characterized as those defective in the pathway of proline biosynthesis from glutamic acid (Glu) to glutamic γ-semialdehyde (GSA), determined genetically by *pro* genes *A* and *B*; Pro$^-$ mutants that possess the metabolic block between Δ^1-pyrroline 5-carboxylic acid (PC), the cyclized product of GSA, and proline, determined genetically by *pro* gene *C*, were never isolated by this method. Further genetic study of *pro* mutants *A* and *B* obtained by 4NPO treatment together with *proC* mutants isolated by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) have provided evidence that *proA*, *proB*, and *proC* genes code for the steps between glutamyl γ-phosphate (GP) and GSA, Glu and GP, and PC and proline, respectively.

We describe in this paper possible mechanisms involved in the phenomenon. Much of the effect of the agent was proved to be selective; 4NPO is more toxic for *pro*$^+$ or *proC*$^-$ cells than for *proA*$^-$ or *proB*$^-$ cells in 4NPO broth

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and selects *proA*⁻ or *proB*⁻ mutants in the culture. As 4NPO showed little mutagenicity on Pro^+ cells, spontaneous mutation of Pro^+ cells to Pro^- (possibly all the *pro* mutants *A*, *B*, and *C*) cells and selection of pro^- *A* and/or *B* mutants thus formed might be the major driving force in the action of 4NPO. Evidence indicating dominance of 4NPO susceptibility over 4NPO resistance of bacteria was also obtained. Besides 4NPO, 4-nitrosopyridine 1-oxide (4NSPO) and 4-hydroxylaminopyridine 1-oxide (4HAPO) but not 4-aminopyridine 1-oxide (4APO) were found to be effective for the selection of Pro^- mutants in the Pro^+ culture. Structural elucidation of metabolic intermediates of 4NPO in the bacterial culture indicated that 4NPO is metabolized, possibly via 4NSPO, to 4HAPO and 4APO. This evidence suggests 4HAPO as a possible proximate compound in the action of 4NPO.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used were *E. coli* K-12 derivatives (Table 1). Pro^- mutants were isolated after treatment of appropriate Pro^+ strains with 4NPO or NTG. Strain W4573 (Pro^+) was kindly supplied by Y. Hirota, Osaka University, Osaka, Japan. The phage strain used for transduction experiments was P1 *vir*.

Materials and media. All the pyridine and quinoline derivatives used in this investigation, i.e., 4NPO (13), 4NSPO (16), 4HAPO (16), 4APO (15), 2NPO (2), 4,4′-azoxypyridine 1,1′-dioxide (15), 4,4′-azopyridine 1,1′ dioxide (AZO) (15), pyridine 1-oxide (PO) (13), 4-nitropyridine (4NP) (5), 4-nitrolutidine 1-oxide (4NLO), (4) 4-nitroquinoline 1-oxide (4NQO) (14) and 4-hydroxylaminoquinoline 1-oxide (4HAQO) (17) (Fig. 1), were synthesized in our laboratory according to the literature.

For 4NPO treatment, Penassay broth (Difco) was used as medium. Media for other experiments have been described previously (8).

Treatment of Pro^+ strains with 4NPO and its derivatives. An overnight culture of Pro^+ strain in Penassay broth was diluted to 10^3 cells/ml in 100 ml of the same broth containing 2 μg of 4NPO per ml or appropriate sublethal concentrations of 4NPO derivatives. After the culture was incubated at 37°C for 20 to 26 h, survivors were plated on EMB/glucose-agar medium, and the colonies grown on the plate were analyzed for proline requirement.

Metabolism of 4NPO by Pro^+ or Pro^- cells. Pro^+ or Pro^- cells were harvested from a 100-ml overnight culture of Davis minimal glucose broth supplemented with 40 μg of L-proline per ml. These cells

TABLE 1. *Bacterial strains of E. coli K-12 used*

Strain	Genetic characteristic[a]	Origin or source
W4573	F⁻ *ara-2⁻ lac-85⁻ gal-2⁻ mal-1⁻ mtl⁻ xyl-2⁻*	
JE2133[b]	As W4573, also *proA1⁻*	W4573
JE2147	As W4573, also *lac⁺*	W4573
JE2149	As JE2133, also *proA1⁺ lac⁺*	JE2133
JE2157	As JE2133, also *proA1⁺*	JE2133
KE113[b]	As W4573, also *proB3⁻*	W4573
KE114[c]	As W4573, also *proC36⁻*	W4573
KE115[c]	As W4573, also *proAB⁻ proC22⁻*	W4573
KE125[c]	As W4573, also *proB17⁻*	W4573
KE126[c]	As W4573, also *proA18⁻*	W4573
KE134[c]	As W4573, also *proC205⁻*	W4573
KE135[c]	As W4573, also *proC106⁻*	W4573
χ646[d]	F′ *proA⁺ proB⁺ lacY⁺/lacY⁻*	(1)
KE343[e]	As JE2133, also F′ *proA⁺ proB⁺ lacY⁺/proA1⁻*	JE2133 × χ646
KE344[f]	As JE2133	KE343
KE345[g]	As JE2133, also F′ *proA⁻ proB⁺ lacY⁺/proA1⁻*	KE343
JE2214	F⁻ *proAB⁻*	
JE1673	F⁻ *proC⁻ ara-2⁻ leu⁻ lac-2⁻ pur⁻ thi⁻ gal-2⁻ mtl⁻ mal⁻ strʳ*	
AB1899NM	F⁻ *lon⁻ thr-1⁻ leu-6⁻ proA2⁻ his-4⁻ thi-1⁻ argE3⁻ ara-14⁻ lacY1⁻ galK2⁻ xyl-5⁻ mtl-1 tsx-33 strA31 sup-37*, nonmucoid	
W3110thy⁻	F⁻ *lpˢ thy⁻*	

[a] Genetic symbols are those used by Taylor and Trotter (21). *proA*⁻ or *proB*⁻ that can grow in minimal medium containing PC but that has not been characterized as either *proA*⁻ or *proB*⁻ is herein tentatively designated *proAB*⁻.
[b] Isolated by 4NPO treatment of W4573 and characterized as *pro*⁻ (8).
[c] Isolated after treatment with NTG followed by penicillin screening and characterized as *pro*⁻ (8).
[d] Kindly supplied by R. Curtiss III (1).
[e] Derived from JE2133 by infection of F *proA⁺ proB⁺ lacY⁺* factor with χ646 as the donor.
[f] Derived from KE343 by elimination of F′ factor by the treatment with acridine orange.
[g] Isolated by 4NPO treatment of KE343.

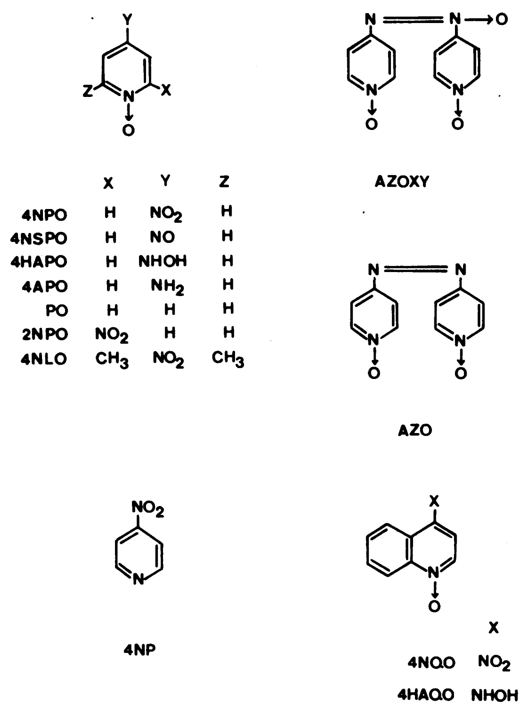

FIG. 1. *Structural features of 4-nitropyridine 1-oxide derivatives used.*

were suspended in 100 ml of Davis minimal glucose broth containing 40 μg of L-proline per ml and 8 μg of 4NPO per ml to give a cell concentration of 2×10^8 cells per ml. The suspension was incubated at 37°C; 2-ml samples were removed at intervals and quickly cooled to 0°C. Then an equivalent volume of ice-cold 10% trichloroacetic acid solution was added to these samples. The mixture was maintained at 0°C for 10 min, and then the ultraviolet spectra of supernatants obtained by centrifugation (3,000 rpm, 10 min) were measured with an Hitachi EPS-2U spectrophotometer using the uninoculated media as a reference solution.

Separation, purification, and identification of metabolites of 4NPO by Pro$^+$ or Pro$^-$ cells. Pro$^+$ or Pro$^-$ cells (ca. 15 g, wet weight) obtained from overnight culture in 1,000 ml of Davis minimal glucose broth containing 40 μg of L-proline per ml were suspended in 1,000 ml of the same broth. A 100-mg portion of 4NPO was added to the suspension, and the mixture was incubated at 37°C on a reciprocal shaker until the absorption maxima shifted to those of expected metabolites of 4NPO, i.e., 280 nm for 4HAPO, 273 nm for 4APO, and 261 nm for 4AP. The suspension was then cooled to 0°C, acidified with 12 N HCl, and centrifuged to separate cells and cellular debris. The supernatant fraction was concentrated to a small volume below 40°C under reduced pressure and was extracted with 99% ethanol. The extract was evaporated below 40°C under reduced pressure to give solid metabolites as the hydrochloric acid salt. If necessary, the crude product was chromatographed on silica gel with ether-methanol (1:1) as eluent. Metabolites thus obtained were converted to their corresponding picrates and recrystallized from water. The structures of metabolites were characterized by mixed melting point determination, elemental microanalysis, and comparison of ultraviolet and infrared spectra and R_f values of thin-layer chromatography with those of authentic specimens.

RESULTS

Reconstruction experiments between Pro$^+$ and Pro$^-$ strains. To investigate the presence or absence of selective toxicity of 4NPO between Prc$^+$ and Pro$^-$ cells, reconstruction experiments were carried out between JE2149 (*pro$^+$ lac$^+$*) and JE2133 (*proA$^-$ lac$^-$*). Overnight cultures (ca. 5×10^8 cells/ml) of *pro$^+$* and *pro$^-$* strains were diluted in Penassay broth with and without 2 μg/ml of 4NPO to give cell concentration of about 10^3 cells/ml. Equal volumes of *pro$^+$* and *pro$^-$* cultures were mixed, the mixture was incubated at 37°C with shaking, and survivors were analyzed at intervals with *lac* as the distinguishing marker. The frequency of production of *pro$^-$ lac$^+$* cells from *pro$^+$ lac$^+$* cells was also determined. Representative results are shown in Fig. 2. In the 4NPO medium, *pro$^-$* cells began to grow after a lag of as long as 3 h, at a slower rate (generation time, ca. 42 min) than cells in broth without 4NPO (ca. 21 min). The viable count of *lac$^+$* (originally *pro$^+$*) cells, on the other hand, began to decrease after the same lag time and reached a minimum cell titer (10^{-2} of the inoculum) after 6 h. Then, the cell titer of survivors began to increase at a similar rate as that of *pro$^-$* cells. The frequencies of *lac$^+$ pro$^-$* cells in the whole *lac$^+$* cells after 5, 6, 8, 10, and 12 h of incubation were found to be 3.7, 37.5, 83.3, 75.9, and 66.5%, respectively. The results indicated that 4NPO exhibits higher toxicity toward *pro$^+$* than *proA$^-$* in the culture. This did not occur in the medium without 4NPO. Similar results were obtained with JE2157 *pro$^+$ lac$^-$* and JE2147 *proA$^-$ lac$^+$*.

Sensitivity of *proA$^-$*, *proB$^-$*, *proC$^-$* and *pro$^+$* cells to 4NPO. Overnight cultures (ca. 5×10^8 cells/ml) of strains W4573 *pro$^+$*, JE2133 *proA$^-$*, KE113 *proB$^-$* obtained by 4NPO treatment, and KE114 *proC$^-$* isolated after NTG treatment were appropriately diluted in Penassay broth containing various concentrations of 4NPO to give cell titer of about 10^3 cells per ml. During incubation at 37°C for as long as 24 h, survivors were counted at certain intervals on EMB-glucose agar. Results are shown in Fig. 3. Sublethal concentrations of 4NPO toward *pro$^+$* and *pro$^-$* were 2.0 μg/ml for *pro$^+$* and *proC$^-$* and 4.0 to 5.0 μg/ml for *proA$^-$* and *proB$^-$*. Figure 4

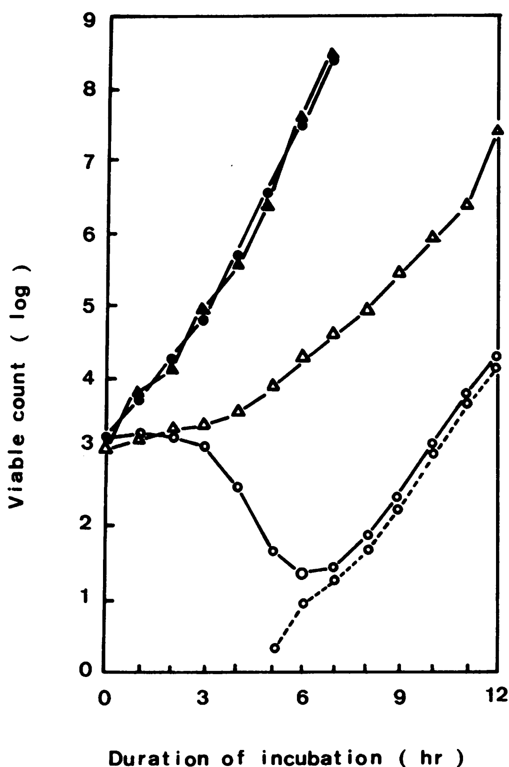

FIG. 2. *Reconstruction experiments between E. coli K12 JE2149 (pro⁺ lac⁺) and JE2133 (proA1⁻ lac-85⁻). Overnight cultures (ca. 5 × 10^8 cells/ml) of pro⁺ and pro⁻ strains were diluted 2 × 10^{-6} in Penassay broth with or without 2.0 μg of 4NPO per ml. Equal volumes of pro⁺ (1.5 × 10^3 cells/ml) and pro⁻ (1.6 × 10^3 cells/ml) cultures were mixed and incubated at 37°C with shaking. Plating with appropriate dilutions at certain intervals was on EMB-lactose-agar. From 20 to 270 lac⁺ colonies were arbitrarily chosen and characterized as pro⁺ or pro⁻ by the replica-plating method. Symbols: ●, viable count of lac⁺ pro⁺ without 4NPO; ▲, viable count of lac⁻ pro⁻ without 4NPO; ○, viable count of lac⁺ pro⁺ plus lac⁺ pro⁻ with 4NPO; △, viable count of lac⁻ pro⁻ with 4NPO; ---○---, viable count of lac⁺ pro⁻ formed in 4NPO medium.*

shows the resulting kinetics for 4NPO treatment (2 μg/ml in Penassay broth) of *pro⁺* and *pro⁻* cells. 4NPO was found to be more toxic for *pro⁺* and *proC⁻* than for *proA⁻* and *proB⁻* cells. Namely, after 6 h of incubation, *proA⁻* and *proB⁻* cells showed about a 1,000-fold greater resistancy to 4NPO than *pro⁺* and *proC⁻* cells. 4NPO sensitivity of *proA⁻*, *proB⁻*, or *proAB⁻ proC⁻* cells obtained by NTG treatment of W4573, KE125, or KE115 was further checked; these *pro⁻* mutants were as resistant to 4NPO as *proA⁻* and *proB⁻* mutants isolated by 4NPO treatment. JE2214 *proAB⁻* and JE1673 *proC⁻*, whose derivation was different than W4573, were resistant and sensitive to 4NPO, respectively. This evidence indicates that whatever the isolation method, *proA⁻* and *proB⁻* mutants are most resistant to 4NPO than *pro⁺* mutants.

To examine 4NPO sensitivity patterns of auxotrophic mutants other than Pro⁻, Met⁻ Pro⁺, His⁻ Pro⁺, and Tyr⁻ Pro⁺ mutants obtained by NTG treatment of W4573 were inoculated into Penassay broth (100 ml) with and without 2.0 μg of 4NPO per ml and incubated at 37°C with shaking. Results indicated that these auxotrophs are as sensitive as W4573 *pro⁺* and *proC⁻* derived from W4573 This observation further supported selection of *proA⁻* and/or *proB⁻* in the *Pro⁺* culture as a possible mechanism for the specific action of 4NPO.

Sensitivity to 4NPO of Pro⁺ revertants and

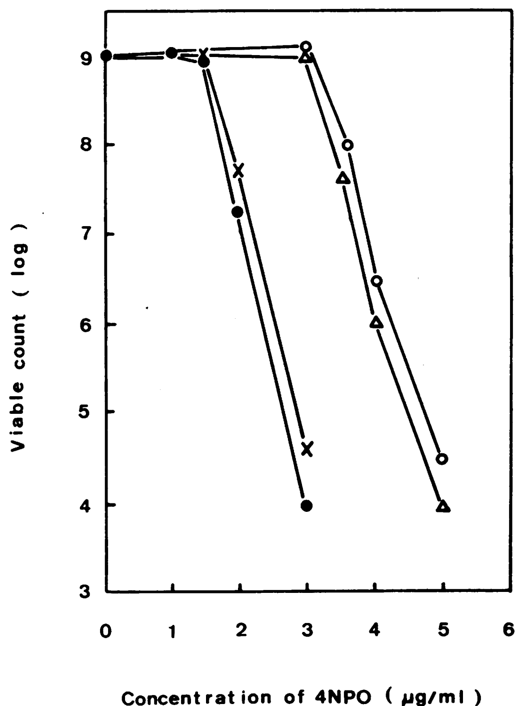

FIG. 3. *Lethal concentration of 4NPO on the viability pro⁺ and pro⁻ cells. Overnight cultures (ca. 5 × 10^8 cells/ml) of pro⁺ and pro⁻ cells were appropriately diluted in Penassay broth (10 ml) containing 1.0 to 6.0 μg of 4NPO per ml to give a cell concentration of about 10^3 cells/ml. After incubation at 37°C for 24 h with shaking, plating with suitable dilution was on EMB-glucose-agar. Symbols: ●, viable count of W4573 pro⁺; ○, viable count of JE2133 proA1⁻; △, viable count of KE113 proB3⁻; ×, viable count of KE114 proC36⁻.*

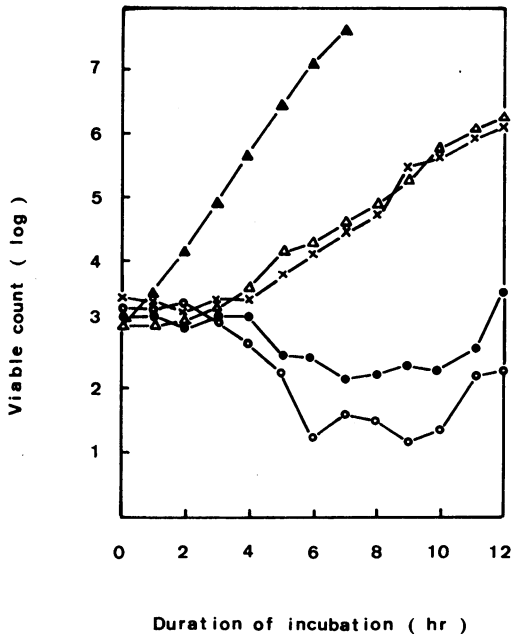

FIG. 4. *Sensitivity of pro+ and pro− cells to 4NPO. W4573 pro+, JE2133 proA1−, KE113 proB3−, and KE135 proC− cells (ca. 10^3 cells/ml) in Penassay broth (100 ml) with or without 2.0 μg of 4NPO per ml were incubated at 37°C with shaking. Symbols: ○, viable count of W4573 with 4NPO; △, JE2133 with 4NPO; ×, KE113 with 4NPO; ●, KE135 with 4NPO; ▲, pro+ and pro− cells tested without 4NPO.*

Pro+ transductants derived from various Pro− strains. To examine whether the observed higher sensitivity to 4NPO of Pro+ cells might be coded by *proA* and/or *proB* genes or some gene(s) linked very closely to these *pro* genes, Pro+ spontaneous revertants and Pro+ transductants were isolated from various Pro− strains, and their 4NPO sensitivity was checked. Spontaneous reverse mutation rates from Pro− to Pro+ were found to be 2×10^{-8} to 5×10^{-10}. Pro+ transductants were obtained with P1 *vir* as transducing phage and W4573 *pro+* as donor strain.

4NPO sensitivity of the Pro+ strains was examined as follows. An overnight culture of Pro+ or Pro− cells to be tested was inoculated with a cell titer of 10^3 cells per ml into Penassay broth containing 2.0 μg of 4NPO per ml. The culture was incubated at 37°C with shaking, and survivors were counted at certain intervals. Strains showing the same kinetics of growth as W4573 *pro+* were classified as 4NPO sensitive ($4NPO^s$), and strains showing the same kinetics of growth of JE2133 *proA−* were classified as 4NPO resistant ($4NPO^r$). Growth response of Pro+ and Pro− strains in Davis glucose minimal medium was also tested.

Results are summarized in Table 2. As expected, KE126 *proA−* and KE125 *proB−* derived from W4573 by NTG treatment were just as resistant to 4NPO as *proA−* and *proB−* strains obtained by 4NPO treatment. Pro+ spontaneous revertants derived from *pro−* strains obtained by NTG treatment, and which could grow in Davis-glucose minimal medium at a rate similar to that of W4573 *pro+*, were found to have recovered 4NPO sensitivity. All Pro+ transductants derived from these *pro−* strains were also found to be 4NPO sensitive. Many of the spontaneous Pro+ revertants derived from *proA−* and *proB−* strains obtained by 4NPO

TABLE 2. *4NPO sensitivity of spontaneous Pro+ revertants and Pro+ transductants derived from Pro− cells*

Strain	Isolation method[a]	Growth responses in D-glucose minimum medium[b]	4NPO sensitivity[c]
W4573 (*pro+*)		++	s
JE2133 (*proA−*)	4NPO	−	r
JE2133 *proA+*	Spont	++	s
		+	r
JE2133 *proA+*	P1 *vir*	++	s
KE113 (*proB−*)	4NPO	−	r
KE113 *proB+*	Spont	+	r
KE113 *proB+*	P1 *vir*	++	s
KE126 (*proA−*)	NTG	−	r
KE126 *proA+*	Spont	++	s
KE126 *proA+*	P1 *vir*	++	s
KE125 (*proB−*)	NTG	−	r
KE125 *proB+*	Spont	++	s
KE125 *proB+*	P1 *vir*	++	s
KE135 (*proC−*)	NTG	−	s
KE135 *proC+*	Spont	++	s
KE135 *proC+*	P1 *vir*	++	s

[a] 4NPO, NTG: isolated from W4573 by 4NPO and NTG treatments, respectivey, and characterized as *proA−*, *proB−*, and *proC−*, Spont: obtained as *pro+* by spontaneous reversion from each *pro−* strains. P1 *vir*: derived from each *pro−* strains by transuction with P1 *vir* and with W4573 as donor strain.

[b] Growth responses in D-glucose minimum medium was classified as follows: ++, same amount of growth as W4573 (*pro+*); +, less growth than original *pro+*; −, no growth.

[c] s, 4NPO-sensitive strain that shows the same kinetics of growth in 4NPO medium as the original W4573 (*pro+*); r, 4NPO-resistant strain that shows the same growth curve in 4NPO medium as JE2133 (*proA−*).

treatment had also recovered equal 4NPO sensitivity as Pro^+ and *proC*$^-$. These results indicated that the gene(s) determining the sensitivity to 4NPO (4NPO^s gene) of bacteria would be in the *proA* or *proB* loci or would be located so close to these *pro* genes that *pro* and 4NPO^s genes could be co-transduced by P1 *vir*.

Dominance of 4NPO sensitivity over 4NPO resistance of *pro* genes. To investigate the dominant or recessive character of *pro* genes for 4NPO sensitivity, the F'*proA*$^+$ *proB*$^+$ plasmid was transferred from χ646 to JE2133 *proA*$^-$, and 4NPO sensitivity of the resulting heterozygote *proA*$^-$/F'*proA*$^+$ *proB*$^+$ was checked. The heterozygote was found to show a similar sensitivity to 4NPO as W4573 *pro*$^+$ (Fig. 5). This F' strain was then treated with acridine orange (6) or sodium dodecyl sulfate (22) to give F^- *proA*$^-$; This F^- had recovered resistance to 4NPO. Similar results were obtained with the heterozygotes of KE113 *proB*$^-$, KE125 *proB*$^-$, or KE126 *proA*$^-$ and F' *proA*$^+$ *proB*$^+$. Furthermore, *proA*$^-$/F' *proA*$^-$ *proB*$^+$ and *proB*$^-$/F' *proA*$^+$ *proB*$^-$ cells derived from the above-mentioned heterozygotes by treatment with 4NPO also recovered the character of 4NPO resistance. The results revealed that the *4NPO*^{s} allele is dominant over the *4NPO*^{r} allele.

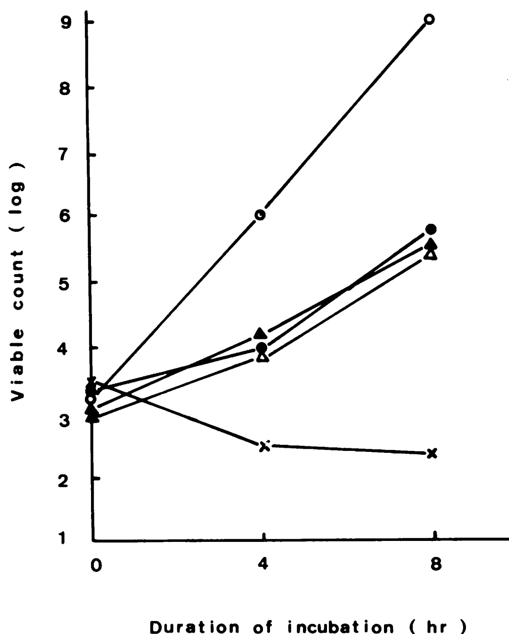

FIG. 5. *Dominance of sensitivity over resistance to 4NPO of pro$^+$ and pro$^-$ strains. JE2133 proA1$^-$, its heterozygote KE343 proA1$^-$/F'proA$^+$ proB$^+$, KE344 proA1$^-$ obtained from KE343 by elimination of F plasmid with acridine orange, and KE345 proA1$^-$/F'proA$^-$ proB$^+$ derived from KE343 by the 4NPO treatment were used. They were incubated in Penassay broth (10 ml) with or without 2.0 μg of 4NPO per ml at 37°C with shaking. Symbols: ●, viable count of JE2133 with 4NPO; ×, viable count of KE343 with 4NPO; ▲, viable count of KE344 with 4NPO; △, viable count of KE345 with 4NPO; ○, viable count of cells being tested without 4NPO.*

Mutagenicity of 4NPO. With respect to possible mechanisms for the action of 4NPO, the mutagenic action of 4NPO on strain W4573 from recessive valine sensitivity (Val^s) to dominant valine resistance (Val^r) (20) was checked. Treatments with 4NQO and ultraviolet irradiation, which are known to exhibit potent mutagenicities on bacteria, were used as control experiments. A refreshed exponential-phase culture (ca. 6×10^8 to 8×10^8 cells/ml) of W4573 Val^s in Penassay broth was centrifuged, and precipitates were washed twice with saline. The cells were suspended in the original volume of Penassay broth, Davis glucose broth, or 0.06 M phosphate buffer (pH 7.2), and 4NPO or 4NQO was added to the suspension to give the desired concentration. After incubation at 37°C for 2 h, 4 ml of sample was centrifuged, washed with 2 ml of saline, and suspended in 1 ml of saline. The suspension was serially diluted and plated on EMB-glucose agar for counting survivors and on DM-glucose agar with 20 μg of L-proline per ml and 40 μg of L-valine per ml for determination of the number of Val^r mutants formed. In preparation for UV irradiation, Val^s cells to be irradiated were suspended in saline instead of Penassay broth.

Representative results are shown in Table 3. 4NPO did not induce any remarkable mutation from Val^s to Val^r, as 4NQO and ultraviolet irradiation had done. It was further shown in other experiments that 4NPO does not induce any remarkable mutation of strain W3110 from *str*^{s} to *str*^{r} nor reverse mutation from JE2133 *proA*$^-$ to *proA*$^+$. Moreover, 4HAPO, a reduced metabolite of 4NPO, did not show potent mutagenicity under those conditions. The results are in accord with previous observations (3).

Rates of spontaneous mutation from Pro^+ to Pro^- cells, which might be correlated to the eventual efficiency of isolation of Pro^- mutants from Pro^+ culture by treatment with 4NPO, were determined by the penicillin-screening method. W4573 *pro*$^+$ cells (2.8×10^8 cells per ml) were incubated in Davis glucose broth containing 300 U of penicillin G per ml at 37°C for 8 h. Whereas the viable count of Pro^+ cells decreased 9.2×10^{-4}, that the Pro^- cells did not under this condition. Survivors grown on EMB-glucose agar were replica plated on Davis glucose agar plates with and without L-pro-

line. Only 2 out of 5,655 colonies tested were Pro⁻ mutants that had the metabolic block between Glu and GSA. Six auxotrophs other than Pro⁻ were also formed. The rate of spontaneous mutation from W4573 *pro*+ to *proA*⁻ and/or *proB*⁻ was calculated to be 3×10^{-7}.

Metabolic conversion of 4NPO by Pro+ and Pro⁻ cells. Possible metabolic conversions of 4NPO by Pro+ and Pro⁻ cells were examined. First, changes of ultraviolet absorption spectra of supernatant fluid of the 4NPO broth inoculated with W4573 *pro*+ cells were examined. Results with *pro*+ cells (Fig. 6) indicated that the ultraviolet absorption maximum of 4NPO at 315 nm shifts to shorter wave length according to the duration of incubation time. Namely, incubation of 4NPO with *pro*+ cells for 12 min caused a decrease of optical density at 315 nm and the appearance of a new peak at 290 nm. After incubation for 18 min, 3 h, and 24 h, the absorption maximum of the supernatant shifted to 280 nm, 275 nm, and 260 nm, respectively. Similar spectral changes were observed when 4NPO was incubated with JE2133 *proA*⁻, KE113 *proB*⁻, or KE135 *proC*⁻, and, furthermore, *pro*+ *chl*⁻ or *proA*⁻ *chl*⁻ cells that were derived from W4573 or JE2133, respectively, and which were nitrate reductase activity-defective mutants. The results suggested that 4NPO might be metabolically reduced to 4HAPO showing $\lambda_{max}^{H_2O}$ 280 nm, 4APO showing $\lambda_{max}^{H_2O}$ 277 nm, and 4AP showing $\lambda_{max}^{H_2O}$ 261 nm.

Next, isolation of metabolites of 4NPO was attempted. A 100-mg portion of 4NPO was added to Davis glucose minimal medium supplemented with L-proline containing about 15 g (wet weight) of Pro+ cells, and the mixture was shaken at 37°C. The ultraviolet absorption maximum of the supernatant fluid was shifted to 280 nm, 277 nm, and 261 nm, respectively, the mixture was cooled to 0°C, and metabolites

TABLE 3. *Mutagenicity of 4NPO toward strain W4573 from valine sensitivity to valine resistance*

Agent	Conc of agents (μg/ml)	Survival (%)	Val^r mutants (per 10^8 survivors)	Relative mutation rate
4NPO	0	100	1.89	1.00
	100	77.4	1.22	0.65
	250	98.1	0.72	0.38
	500	44.0	1.25	0.66
	750	55.3	2.82	1.49
	1,000	42.7	1.85	0.98
4NQO	2.5	72.9	6.46	3.42
	5.0	99.4	6.50	3.44
	7.5	46.8	20.2	10.7
	10	41.2	25.0	13.2
UV irradiation[b]	0[c]	100	2.88	1.00
	10	23.9	9.74	3.4
	20	17.1	27.0	9.4
	30	4.74	77.3	26.8

[a] The inoculum was 6.4×10^8 cells/ml in Penassay broth. Incubation was carried out at 37°C for 2 h.
[b] The inoculum was 1.7×10^8 cells/ml.
[c] Time of irradiation (seconds).

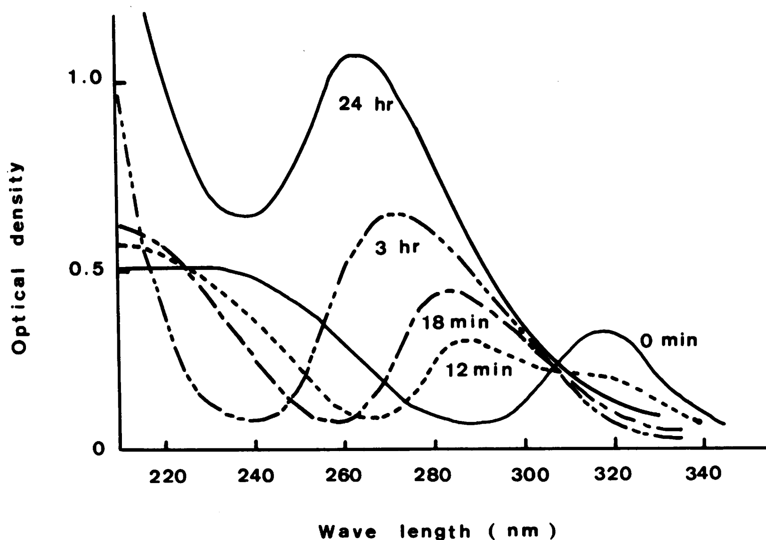

FIG. 6. *Changes of ultraviolet absorption spectrum of 4NPO in Pro+ culture. For procedure, see Materials and Methods. Absorption maxima of authentic specimens were as follows: 4NPO, 315 nm; 4HAPO, 280 nm; 4APO, 277 nm; 4AP, 261 nm.*

were extracted and purified as described above. The structures of metabolites thus isolated were characterized as 4HAPO, 4APO, and 4AP, respectively (Table 4). A discrepancy between melting points of a metabolite characterized as 4APO and its authentic specimen was observed; the metabolite may not have been fully purified from some unknown contaminants that had little effect on the other properties of 4APO. The results indicated that the reduction of the nitro group of 4NPO proceeds equally in Pro$^+$ and Pro$^-$ cultures, and that the further reduction of the *N*-oxide bond also occurs (Fig. 7).

Metabolic reduction of 4NPO in Pro$^+$ culture under anaerobic condition was further carried out. Results indicated that 4NPO can be reduced at a 2.2 times faster rate than that observed under aerobic conditions.

The metabolic conversion process of 4NPO by JE2133 *proB*$^-$ cells in synthetic minimal medium was also investigated. 4NPO was found to be reduced to 4HAPO and 4APO as in Penassay broth.

Structural modification of 4NPO and elective isolation of Pro$^-$ mutants. It has been shown that 4NPO is reduced by Pro$^+$ and Pro$^-$ cells to 4HAPO, 4APO, and 4AP. To investigate which metabolite might be a proximate compound or a compound most close to it for the phenomenon, W4573 *pro*$^+$ was incubated in Penassay broth with each 4NPO derivative of the series shown in Fig. 1. Representative results are shown in Table 5. Isolation of Pro$^-$ mutants was observed with 4NSPO (12 μg/ml) and 4HAPO (40 μg/ml) but not with AZOXY, AZO, 4APO, PO, and 2NPO. The results suggest that the nitro, nitroso, or hydroxylamino group at the 4 position and the *N*-oxide moiety

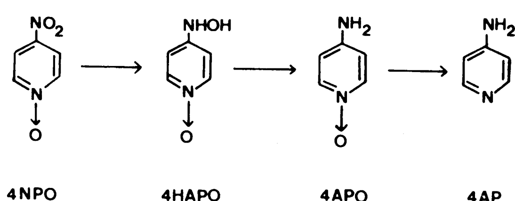

FIG. 7. *Metabolic conversion process of 4NPO in bacterial culture.*

TABLE 5. *Action of 4NPO derivatives on W4573 (Pro$^+$) strain leading to the isolation of Pro$^-$ mutants*[a]

Compound	Concn of agent (μg/ml)	Viable count (cells/ml)	Frequency (%) of Pro$^-$ colonies
4-Nitropyridine 1-oxide (4NPO)	0		0
	2		100
4-Nitrosopyridine 1-oxide (4NSPO)	12	6.4×10^4	10.6
4-Hydroxylamino-pyridine 1-oxide (4HAPO)	90	1.3×10^5	30.8
	100	8.6×10^3	11.0
4,4'-Azoxypyridine 1,1'-dioxde (AZOXY)	50	1.4×10^3	0
4,4'-Azopyridine 1,1'-dioxide (AZO)	40	5.2×10^2	0
4-Aminopyridine 1-oxide (4APO)	500	2.2×10^9	0
4-Nitropyridine (4NP)	25		0
Pyridine 1-oxide (PO)	1,000	4.0×10^9	0
2-Nitropyridine 1-oxide (2NPO)	12.5	1.4×10^2	0
4-Nitrolutidine 1-oxide (4NLO)	20	4.0×10^6	0.3
	25	1.6×10^3	95.7
4-Nitroquinoline 1-oxide (4NQO)	0.8	1.7×10^8	29.6
4-Hydroxylamino-quinoline 1-oxide (4HAOO)	40	4.2×10^9	2.0

[a] Incubation of strain W4573 (Pro$^+$) with each compound was carried out in Penassay broth (100 ml) at a cell concentration of 10^3 cells/ml at 37°C for 24 h.

TABLE 4. *Characterization of metabolites of 4NPO by pro$^+$ and pro$^-$ cells*

Compounds	Source	Melting point (°C)[a] (decomp.)	Ultraviolet spectra $\lambda_{max}^{H_2O}$ (nm)	Ultraviolet spectra ϵ[b]	Elemental analysis C	Elemental analysis H	Elemental analysis N	Yield (%)
4HAPO Picrate	Metabolic product	172–174	280 358	21,700 14,600	37.59	2.63	20.21	21
	Authentic specimen	172–174	280 358	23,200 14,800	37.19	2.55	19.72	
4APO Picrate	Metabolic product	189–194	275 358	21,800 15,200	38.85	2.67	20.44	10
	Authentic specimen	204–206	277 358	21,400 13,800	38.95	2.67	20.65	
4AP Picrate	Metabolic product	218–220	260 358	22,200 12,800	40.53	2.75	21.03	25
	Authentic specimen	217–218	261 358	22,200 13,200	40.87	2.81	21.67	

[a] Melting points were taken on a kofler-type hot stage, and are uncorrected.
[b] Molecular intensity.

at the 1 position on the pyridine (and possibly quinoline) skeleton are indispensable for the action of the agent.

It is noted that 4NPO is more efficient than 4NQO in such action, and that every agent acts on Pro^+ cells most efficiently at a sublethal concentration of the agent.

Reconstruction experiments with these active 4NPO derivatives, i.e., 4HAPO, 4NSPO, 4NLO and 4NQO, were carried out between JE2149 *pro^+ lac^-* and JE2133 *$proA^-$ lac^+* and showed higher toxicity of the compounds toward *pro^+* cells than *pro^-* cells, as was the case with 4NPO.

Bacteriostatic action of 4NPO toward pro^+ cells in minimal medium. As mentioned above, the bacteriocidal action and selective toxicity of 4NPO toward *pro^+* cells were observed in a complete medium, Penassay broth. To check further the mechanisms of the specific action of the agent, reconstruction experiments of the 4NPO treatment between JE2149 (*pro^+ lac^+*) and KE113 (*$proA^-$ lac^-*) JE2133 (*$proB^-$ lac^-*), or KE134 (*$proC^-$ lac^-*) were carried out in a synthetic minimal medium, Davis glucose minimal medium, supplemented with 20 μg of L-proline per ml. Results (Fig. 8) indicated that whereas 4NPO at a concentration of 2.5 μg/ml acted on *pro^+* cells bacteriostatically for as long as 35 h, the agent allowed the growth of *$proA^-$*, *$proB^-$*, and *$proC^-$* cells, although with longer generation times compared with those without the agent. Similar results were obtained when each strain was treated independently with 4NPO. When the minimum medium further supplemented with 0.1% Casamino Acids was used, however, *proC* cells showed 4NPO sensitivity, similar to *pro^+* cells.

The observation of bacteriostatic action of 4NPO on *pro^+* cells in synthetic minimal medium led to an examination of morphological features of *pro^+* cells in the medium. An overnight culture of W4573 (*pro^+*) with a cell concentration of 10^3 cells per ml was incubated in 1 liter of Davis glucose minimal medium containing 40 μg of L-proline per ml and 2.5 μg of 4NPO per ml. After incubation with shaking for 24 h, the culture was filtered with suction through a Sartorius membrane filter (pore size, 0.45 μm). The cells collected on the filter were suspended in 1 ml of Davis glucose minimal medium. The nuclear apparatus of the cells was stained with Giemsa solution (Merck) after the cells were treated with 1 N hydrochloric acid at 60°C for 5 min to remove ribonucleic acid. Observation with a phase-contrast microscope (Nikon) revealed that *pro^+* cells exposured to 4NPO had formed long nonseptate multinucleate filaments, the longest ones being 312 μm in length. Nuclear materials were distributed throughout the length of these filaments. Filament formation of *pro^+* cells was not observed in the medium without 4NPO. The average cell length per nucleus of 30 long filamentous *pro^+* cells was 3.9 μm, and that of the normal cells growing in the medium without 4NPO was 1.4 μm. Formation of filamentous cells, however, was not observed when JE2133 (*$proA^-$*) cells were incubated with and without 2.5 μg of 4NPO per ml.

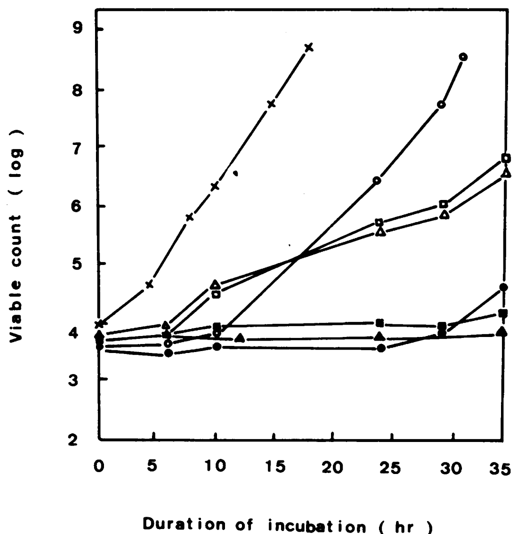

FIG. 8. *Reconstruction experiments between JE2149 (pro^+ lac^+) and JE2133 ($proA1^-$ lac-85^-), KE113 ($proB3^-$ lac-85^-), or KE134 ($proC^-$ lac-85^-) in Davis-glucose minimal medium containing 40 μg of L-proline per ml. Overnight cultures of pro^+ and pro^- strains were diluted to give cell concentration of ca. 10^4 cells/ml in Davis glucose minimal medium containing L-proline with or without 2.5 μg of 4NPO per ml. Equal volumes of pro^+ and pro^- cultures were mixed and incubated at 37°C with shaking. Plating with suitable dilutions was EMB-lactose-agar. Symbols: □, viable count of JE2133 with 4NPO; △, viable count of KE113 with 4NPO; ○, viable count of KE134 with 4NPO; ■, ▲, ●, viable count of JE2149 with 4NPO; ×, viable count of pro^+ and pro^- cells without 4NPO.*

As control experiments, UV irradiation of AB 1899NM (*lon^-*) strain and thymine starvation of W3110 *thy^-* strain were carried out, and the filamentous cells formed were analyzed by the nuclear staining method. The average cell length per nucleus was 1.2 μm in the filaments formed by the UV irradiation method and 2.7 μm in those isolated after thymine starvation. These results suggested that the action of 4NPO on *pro^+* cells in minimal medium might

be similar to the effect of thymine starvation on *thy*$^-$ cells (inhibition of deoxyribonucleic acid biosynthesis) rather than the effect of UV irradiation on *lon*$^-$ cells (inhibition of septum formation).

When filamentous cells derived from *pro*$^+$ cells by treatment with 4NPO were transferred into fresh minimal medium without the agent, they immediately began to regrow with the same kinetics of growth as *pro*$^+$ cells in the medium without 4NPO; the frequency of filamentous cells among total cells decreased by one-half after incubation for one generation time (data not shown).

DISCUSSION

We have found that 4NPO can act on *E. coli* K-12 Pro$^+$ strains in Penassay broth, leading to highly efficient isolation of Pro$^-$ mutants (7, 8). The Pro$^-$ cells thus isolated were *proA*$^-$ and/or *proB*$^-$ deficient in the biosynthetic pathway of proline, i.e., the process from Glu to GSA. With respect to possible mechanisms involved in the action of 4NPO, reconstruction experiments between *pro*$^+$ and *proA*$^-$ cells indicated that 4NPO is more toxic for *pro*$^+$ cells than for *proA*$^-$ cells (Fig. 2). Additional reconstruction experiments between *pro*$^+$ and *proB*$^-$, together with experiments on the bacteriocidal action of 4NPO on *pro*$^+$, *proA*$^-$, *proB*$^-$, and *proC*$^-$ cells in Penassay broth, further confirmed the higher sensitivity of *pro*$^+$ and *proC*$^-$ than *proA*$^-$ and *proB*$^-$ toward 4NPO (Fig. 3 and 4). Auxotrophic mutants other than *pro*$^-$ were found to have similar 4NPO sensitivity as *pro*$^+$ cells. These data indicate that the possible mechanism for the specific action of 4NPO may be the selective toxicity of the agent between *pro*$^+$ and *proA*$^-$ or *proB*$^-$ but no *proC*$^-$ cells. The fact that only *proA*$^-$ and *proB*$^-$ cells could be isolated by treatment of Pro$^+$ cells with 4NPO is in accord with the mechanism suggested.

Possible correlations between "4NPO-sensitive gene(s)" and *proA* or *proB* genes were then studied (Table 2). All *pro*$^+$ transductants derived from *proA*$^-$ and *proB*$^-$ cells with P1 *vir* phage and W4573 *pro*$^+$ as donor recovered sensitivity to 4NPO. Spontaneous Pro$^+$ revertants derived from *proA*$^-$ and *proB*$^-$ cells, on the other hand, were classified into two types, i.e., those fully growing in Davis glucose minimal medium and showing sensitivity to 4NPO and those growing rather slowly in the medium and showing resistance to 4NPO (data not shown). The latter type of *pro*$^-$ mutants may be those under the suppression of proline requirement by mutation of arginine requirement (11). These results suggest that *4NPO*^{s} gene(s) should be on or very close to the *proA* and *proB* loci and that both products of the *proA* and *proB* genes may be involved in the observed sensitivity of bacteria to 4NPO. The results of the dominance test using *pro*$^-$/F′ *proA*$^+$ *proB*$^+$ heterozygotes revealed that the 4NPO-sensitive allele is dominant over the 4NPO-resistant allele (Fig. 5). This suggests that gene product(s) of *proA* and/or *proB* may be associated in some way with the 4NPO sensitivity of Pro$^+$ cells, for example, by interference with 4NPO or more likely with its metabolite(s) to form a toxic substance(s) for the bacterial cell.

4NPO was found not to induce any remarkable mutation on strain W4573 (Table 3). This indicates that *proA*$^-$ and *proB*$^-$ cells formed from *pro*$^+$ cells by spontaneous mutation might be selected by 4NPO. The fact that the rate of spontaneous mutation from *pro*$^+$ to *proA*$^-$ and/or *proB*$^-$ was 3×10^{-7} may be in agreement with an already observed large fluctuation of frequency of *pro*$^-$ production by 4NPO treatment (8).

Considering 4NPO as an analogue of proline (10), the following answers to the question of why *proA*$^-$ and *proB*$^-$ cells show resistance to 4NPO are possible. First, *pro*$^+$ and *proC*$^-$ cells may incorporate much more 4NPO as a toxic substance than *proA*$^-$ and *proB*$^-$ cells do. This explanation was disproved by experiments incorporating [^{3}H]4NPO into the *pro*$^+$ and *pro*$^-$ cells; no significant difference between the incorporation rates of ^{3}H was observed between *pro*$^+$ and *pro*$^-$ (Inuzuka and Tomoeda, unpublished data). A second possible explanation is that whereas *pro*$^+$ and *proC*$^-$ cells are less efficient in metabolizing 4NPO as a detoxication process, *proA*$^-$ and *proB*$^-$ cells can metabolize 4NPO at a higher rate. Again, experiments on metabolic conversion of 4NPO with bacterial cells showed no significant differences in reduction rates of the agent to 4HAPO and 4APO by *pro*$^+$, *proA*$^-$, *proB*$^-$, or *proC*$^-$ cells. Moreover, the reducing enzyme responsible for the metabolism of 4NPO may be DT diaphorase, as reported in the case of metabolic reduction of 4NQO (18). This further suggests that the second explanation is unlikely. And third, intermediate(s) of proline biosynthetic pathway between Glu and Pro, which can be synthesized in *pro*$^+$ and *proC*$^-$ but not in *proA*$^-$ and *proB*$^-$, may be in some respect associated with the toxicity of 4NPO toward bacterial cells. With respect to this question, additional effect of PC on the toxicity of 4NPO toward bacterial cells was examined. However, no effect of PC at concentrations as high as 500 μg/ml in Penassay broth or Davis glucose minimal medium containing L-proline was observed.

It is interesting to note that 4NPO acted bacteriostatically on pro^+ cells but not on pro^- cells in minimal medium, leading to the formation of long nonseptate multinucleate filamentous cells. Comparison of the results with those of filament formation of lon^- cells with UV irradiation and of thy^- cells by thymine starvation suggested that 4NPO-induced formation of filamentous cells might result from possible inhibition of DNA synthesis of bacterial cells by the agent. However, further experiments should be conducted before any decisive explanation can be given.

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