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Studies on Development of Resistant Strains to Antibiotics and Antituberculosis Agents(II) Isolation of Rifampicin Resistant Mutants from *Clostridium butyricum*

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Abstract □ The preparation of *Clostridium butyricum* is used as a normalizing agent for human intestinal flora. When the microbe is simultaneously used with rifampicin, it is inactivated by the antibiotic. To develop rifampicin-resistant mutants, rifampicin-sensitive strain Miyairi II 588 of *C. butyricum* was treated with nitrosoguanidine (NTG). To ensure stable resistance to rifampicin, we examined whether the resistance was plasmid-mediated or chromosome-mediated. It was found that the resistance of four mutant strains was not mediated by its inherent plasmid, but by the chromosomal mutation. These strains were examined for the susceptibility and resistance to other antituberculosis agents and antibiotics. The results showed that these mutants were resistant to the high concentration of the antituberculosis agents.

Keywords ■ *Clostridium butyricum,* rifampicin-resistant mutant, NTG, ethidium bromide, chromosome mediated resistance, resistance induction, MIC, transformation.

After Miyairi reported first that *Clostridium butyricum* was antagonistic to pathogenic microorganisms of intestinal flora,[1] Mose and Mose found that it was inhibitory against Ehrlich tumor[2] and Yoshitani observed its therapeutic effects on acute and chronic intestinal infections by combined use with anticonvulsives and sedatives. Hara *et al.* found that it was prophylactic against dysentery.[3] Kobayashi and Tanami reported that it promoted the growth of the beneficial microorganisms such as *Lactobacillus bifidus* and *L. acidophillus* among those that were found in the intestinal flora.[4] Based on these reports, the preparation of *Clostridium butyricum* has been used for treatment or prophylaxis of intestinal diseases.

In our previous report,[5] we examined the preparations of butyric and lactic acid bacteria for resistance against antituberculosis drugs and antibiotics that are currently being used in Korea and found that the preparation of *Clostridium butyricum* was highly susceptible to rifampicin as well as penicillins, cephalosporins and some of other antibiotics, indicating that concomitant oral administration of these antibiotics with the butyric acid bacteria may destroy the bacteria. Since it was necessary to develop resistant strains of *Clostridium butyricum* to rifampicin which is usually employed on a long-term basis for chronic diseases, we prepared rifampicin resistant mutants with nitrosoguanidine (NTG).

The resistant mutants were examined for whether they were plasmid-mediated or chromosome-mediated by using, a plasmid-curing agent, ethidium bromide. When minimum inhibitory concentrations of nine anti-tuberculosis drugs and 19 antibiotics were determined for these mutants, it was shown that these mutants were resistant to rifampicin.

EXPERIMENTAL METHODS

1. Microorganisms

The strain used in this study was *Clostridium butyricum* Miyairi II 588 which was found to be susceptible to rifampicin by the authors, as shown in Table I. *Escherichia coli* used in the present study was supplied by College of Medicine, Seoul National University.

2. Media

The culture medium for strain Miyairi II 588 was modified fluid thioglycollate(MFT) medium.[6] The medium for *E. coli* was nutrient broth(NB)

Table I. MIC of antituberculosis agents against *Clostridium butyricum*

Antituberculosis agents	MIC (μg/ml)
Streptomycin	200 <
Kanamycin	100 <
Rifampicin	0.05
Capreomycin	50
Ethambutol	100 <
INAH	100 <
Cycloserine	100 <
Pyrazinamide	100 <
Prothionamide	100 <

Table II. Culture Media (unit: g/l)

MFT medium		NB medium		EMB agar	
Fluid thioglycollate	29.8	Beef-extract	3.0	Peptone	10.0
Glucose	10.0	Peptone	5.0	Lactose	10.0
Tween 80	1.0			K_2HPO_4	2.0
Ammonium citrate	2.0			Eosin Y	0.4
Sodium acetate	*5.0*			*Methylene-* blue	*0.065*
$MgSO_4 \cdot 7H_2O$	0.1			Agar	18
$MnSO_4$	0.05				
K_2HPO_4	2.0				
$FeSO_4$	0.034				
KH_2PO_4	6.0				
Sodium thioglycollate	1.0				

medium. To select transformed strains of *E. coli*, EMB agar medium was used(Table II).

3. *Reagents*

A) Mutagen: *N-methyl-N'*-nitro-N-nitrosoguanidine(MNNG or NTG) of Nakarai Chemicals Co. was used.

B) Curing agent: ethidium bromide of Aldrich Chemical Co. was used.

C) Anti-tuberculosis drugs and antibiotics: they were supplied by the Central Research Labs, Dong-A Pharm. Co., Seoul and are presented in Table III.

D) Anaerobic condition: to keep media and buffers anaerobic, sodium thioglycollate of Sigma Chemical Co. was used.

Table III. Antituberculosis agents and antibiotics

Agents	Penicillins	Cephalosporins	General antibiotics
Strepto mycin	Ampicillin	Cephazolin	Minocycline
Kanamycin	Penicillin V	Cephaloridine	Chloramphenicol
Rifampicin	Amoxycillin	Cephatrizine	Erythromycin
Capreomycin	Cloxacillin	Cephalexin	Gentamicin
Ethambutol	Metampicillin	Ceptizoxime	
INAH	Carbenicillin	Cefamandole	
Cycloserine		Cephadroxil	
Pyrazinamide		Cefuroxime	
Prothionamide		Moxalactam	

4. *Screening of rifampicin resistant mutants*

Mutagenesis by NGT was conducted according to the modified methods of Schwartz *et al.*[7] and Thompson *et al.*[8] as shown in Fig. 1.

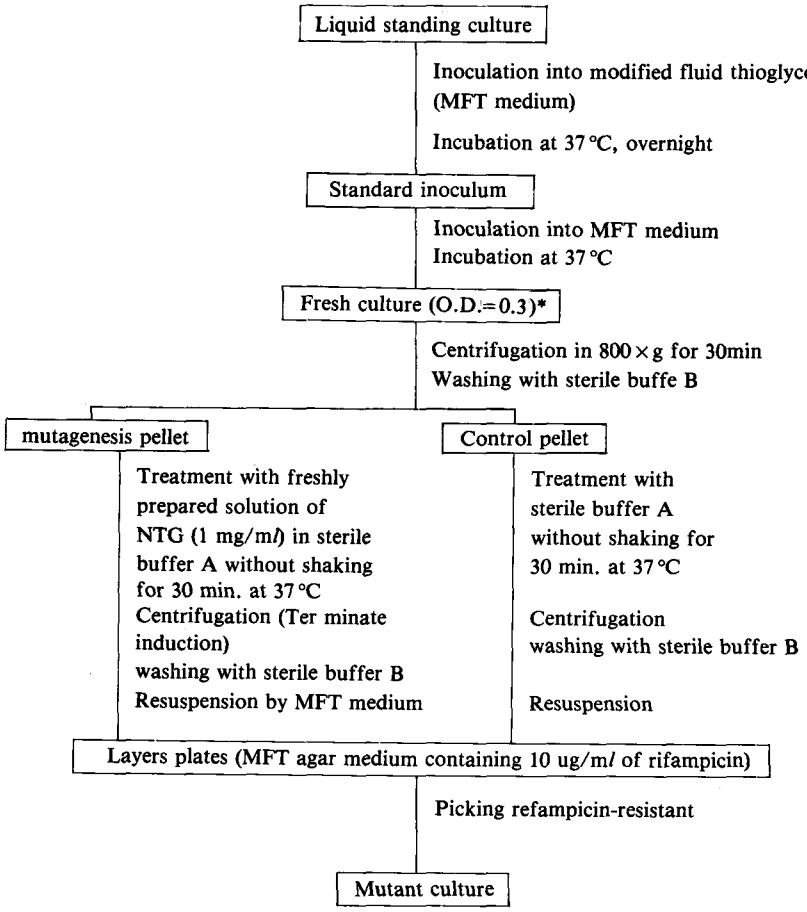
A) Preparation of inoculum: the parent strain grown overnight in MFT medium at 37 °C was used as standard inoculum.

B) Fresh culture: the culture that grew to the point of optical density(=O.D.) 0.3 at 540 nm in MFT medium was used.

C) *Mutagenesis:* the supernatant of the fresh culture was removed by centrifugation at 800 × g for 30 min. The pellets were washed by 50 mM potassium phosphate buffer(pH 6.8) containing 0.1%(w/v) sodium thioglycollate and were divided into mutagenesis and control groups.

For the mutagenesis group, the pellets were shaken in a sterile solution of 0.1% NTG in 50 mM potassium phosphate buffer(pH 6.8, buffer A) and cultured at 37 °C for half an hour. The mutagenesis was terminated by removing the NTG solution after centrifugation. After washed with buffer B, the pellets were resuspended in MFT medium, and 0.1 ml and 1 ml of this medium were added to 200 ml of MFT agar medium and 200 ml of the agar medium containing 10 ug/ml of rifampicin, respectively. They were plated out in six Petri dishes and incubated at 37 °C for three to seven days. Rifamipicin resistant colonies were observed, and 25 of them were transferred to MFT medium and subcultured for seven times for two weeks, thereby allowing them enough time for back mutation if any. These were used as mutant culture.

After the control pellets were shaken only in buffer A, they were incubated at 37 °C for 30 min. After the centrifugation, they were treated in the same manner as the mutagenesis pellets to observe whether spontaneous mutation occurred or not.



**O.D. was measured at 540 nm.
Buffer A: 50 mM potassium phosphate buffer (pH 6.8)
Buffer B: Buffer A containing 1g of soldium theioglycollate per liter.

Fig. 1. Screening of rifampicin resistant mutants by treatment with nitrosoguanidine (NTG) in *Clostridium butyricum* Miyairi II 588.

The porcedure was shown in Fig. 1.

D) Selection of resistant mutants: after standard mutant suspension was prepared from the mutant culture, it was diluted to 1:200 in MFT medium to obtain cell broth. This was mixed with the same volume of MFT medium containing 50 ug/ml rifampicin. The mutants that survived against 100 ug/ml of MIC were selected as potential resistant strains.

The method of MIC determination of the resistant mutants against the various drugs was shown in Fig. 2.

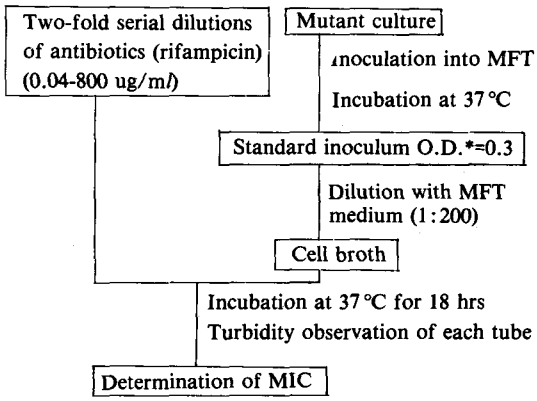


Fig. 2. Determination of MIC of antituberculosis agents and antibiotics against mutants of *Clostridium butyricum*.

5. *Plasmid-curing test by ethidium bromide (EB)*

This test was conducted to ensure that the drug resistance was mediated by chromosome but not by inherent plasmid. According to the modified

method of Bouanchaud *et al.*,[9] cell broth was prepared by diluting the standard cultures of nine resistant strains to 1:200 in fresh medium. After it was mixed with the same volume of two-fold serial dilutions of EB, the mixture was incuabated at 37 °C. To determine subinhibitory concentrations of EB, OD of the cell broth was measured at 620 nm and percent of the resistant strains was calculated from OD. After they were diluted in buffer B, it was plated in MFT agar medium to produce colonies. Among these colonies, 100 colonies were respectively inoculated to MFT medium. Their growth was tested at the four-fold dilution of the MIC of the resistant strain and at the four-fold higher concentration of its MIC, to ascertain whether the resistance was still retained. These 50 strains retaining the resistance were again tested in the same manner (Fig. 3).

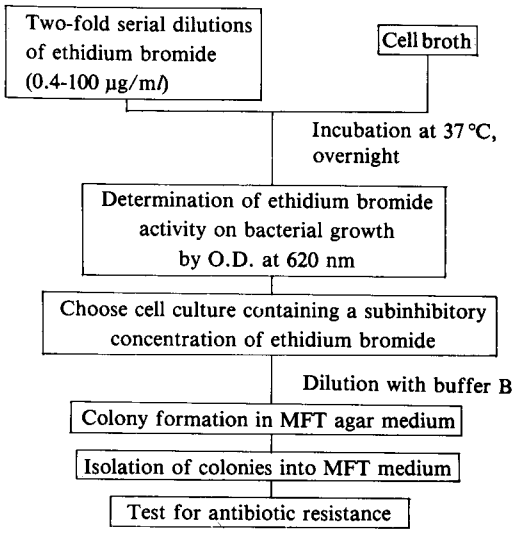


Fig. 3. Elimination of the resistance in the resistant strains by ethidium bromide

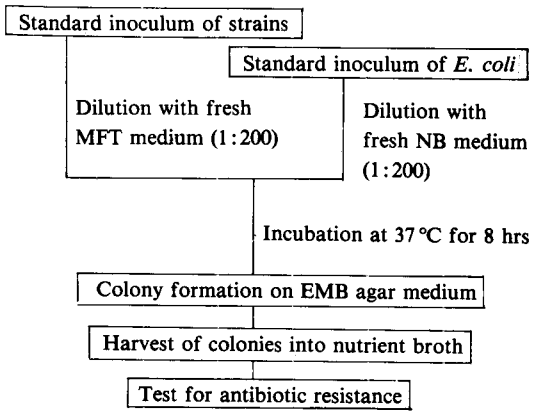


Fig. 4. Procedure of transformation

6. Transformation test

The standard cultures of nine resistant strains were diluted into fresh MFT medium at the ratio of 1:200. The standard culture of *E. coli* was diluted into fresh NB medium at the same ratio. After these were mixed, they were incubated at 37 °C for eight hours (after eight hours *E. coli* was gradually destroyed by the strains 588). Then colonies were formed in EMB agar medium that selectively allows gram-negative bacteria to grow. Their MICs against rifampicin were measured and compared with those of their parent strains to observe whether transformation might occur in *E. coli* (Fig. 4).

7. Resistance test to anti-microbial agents

Four strains of the chromosome-mediated resistant mutants of Miyairi II 588 were tested against those drugs listed in Table III.

RESULTS AND DISCUSSION

1. Isolation of rifampicin resistant mutants from strain Miyairi II 588

In the mutagenesis pellets that were treated by NTG, the survival rate of the strain was 7.4×10^2 colonies/m*l*, and that of the mutant strain was 8.3 colonies/m*l*. In the cotrol pellets, the rate of the strain 588 was 8.3×10^3 colonies/m*l* and that of the mutant strain was zero, respectively. To evaluate conditions for mutation, survival ratio (=SR, %) and mutagenesis ratio (=MR, %) were calculated according to the equations as shown in Table IV.

Table IV. Survival ratio (S.R., %) and mutagenesis ratio (M.R., %)

	Mutagenesis	Control
No. of viable colonies/m*l*	7.4×10^2	8.2×10^3
No. of mutant colonies/m*l*	8.3	0

$$\text{S.R.} - \frac{\text{No. of viable colonies of mutagenesis}}{\text{No. of viable colonies of control}} \times 100 = 9.0\%$$

$$\text{S.R.} - \frac{\text{No. of mutant colonies of mutagenesis}}{\text{No. of viable colonies of mutagenesis}} \times 100 = 1.1\%$$

Table V. MIC (ug/m*l*) of rifampicin against nine resistant strains among 25 mutants

Strain No.	6	16	17	18	21	22	23	24	25
MIC (ug/m*l*)	400	400	200	200	100	100	200	100	400

Since no mutant colony appeared in the case of the control pellets, the mutation to resistance in the mutagenesis pellet group was apparently caused by NTG, but not by spontaneous mutation. The SR value was 9.0% and the MR value was 1.1% so that the conditions for mutagenesis appeared to be optimum. Among the 25 mutant strains isolated, nine potential strains were selected and determined to have more than 2000-fold increase in the resistance to rifampicin (Table V).

2. *Ascertainment of resistance by plasmid-curing test*

To ascertain whether the drug resistance of the nine mutants was due to chromosome or plasmid, the putative plasmid in the resistant strains was removed by treating with EB and then tested whether they still retained the resistance or not.

According to the following equation, ratio of ethidium bromide resistant organism (ERO) was calculated with OD of the cultures at the various concentrations of EB.

E.R.O. (%) =

O.D. of bacterial culture broth containing ethidium bromide-O.D. of blank broth / O.D. of bacterial culture broth-O.D. of broth-O.D. of blank broth

The results were shown in Figs. 5 and 6. On the basis of the data, the sub-MIC values of EB for the nine strains were determined. By use of the sub-MIC, the numbers of resistant colonies were compared with those of sensitive colonies. As shown in Table VI, four strains (Nos. 6, 22, 23, and 25) were found to possess chromosome-mediated resistance and five strains (Nos. 16, 17, 18, 21, and 24) appeared to have plasmid-mediated resistance. The rate of loss of the resistance was 16-33%, the median value being 20%. These results showed that at least in the four strains resistance mutation occurred on the chromosome of the bacteria.

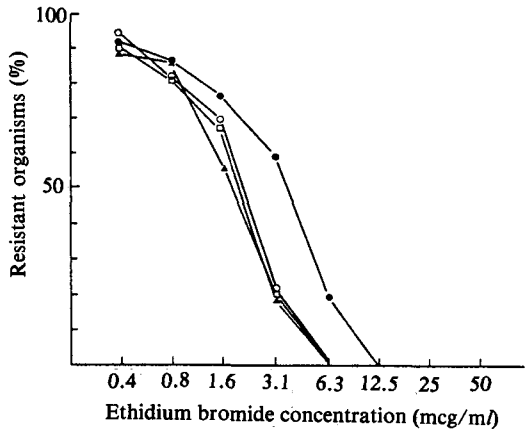


Fig. 5. Determination of ethidium bromide activity, on growth of the strains (strain 6: ○ – ○ , strain 16: △ – △ , strain 17: ● – ● , strain 18: □ – □)

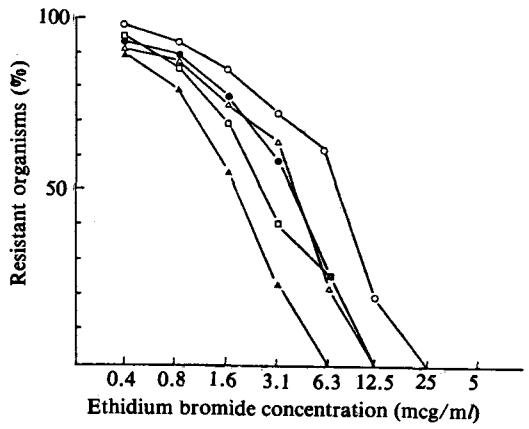


Fig. 6. Determination of ethidium bromide activity on growth of the strains (strain 21: △ – △ , strain 22: ▲ – ▲ , strain 23: ● – ● , strain 24: □ – □ , strain 25: ○ – ○)

3. *Transformation test*

Table VI. Sub-MIC of ethidium bromide against nine resistant strains and the frequency of elimination of rifampicin resistance by ethidium bromide

(μg/ml)

Strain No.	6	16	17	18	21	22	23	24	25
Sub-MIC	3.1	3.1	6.3	3.1	6.3	3.1	6.3	6.3	12.5
No. of tested total colonies	150	100	100	100	100	150	150	100	150
No. of resistant colonies	150	79	67	76	81	150	150	84	150
No. of sensitive colonies	0	21	33	24	19	0	0	16	0
Elimination frequency (%)	0	21	33	24	19	0	0	16	0

Table VII. MIC of rifampicin agains nine transformed *E. coli* strains

(μg/ml)

Transformed strain	×6	×16	×17	×18	×21	×22	×23	×24	×25	Ref.
MIC (μg/ml)	6.3	6.3	6.3	25	50	6.3	6.3	50	6.3	6.3

Table VIII. MIC (ug/ml) of antituberculosis agents against four chromosome-mediated resistant strains of *Clostridium*

Agents \ Strain No.	6	22	23	25	Parent
Streptomycin	200	200	200	200	200
Kanamycin	100	100	100	100	100
Rifampicin	400	100	200	400	0.05
Capreomycin	200	200	100	200	50
Ethambutol	100	100	100	100	100
INAH	100	100	100	100	100
Cycloserine	100	100	100	100	100
Pyrazinamide	100	100	100	100	100
Prothionamide	100	100	100	100	100

Table IX. MIC (mg/ml) of penicillins aganist four chromosome-mediated resistant strains of *Clostridium butyricum*

Penicillins \ Strain No.	6	22	23	25	Parent
Ampicillin	0.2	0.4	0.2	0.4	0.05
Penicillin V	0.2	0.2	0.2	0.2	0.2
Amoxycillin	0.1	0.1	0.1	0.1	0.1
Cloxacillin	3.1	6.3	6.3	6.3	3.1
Metampicillin	0.4	0.4	0.4	0.4	0.05
Cabenicillin	1.6	3.1	3.1	3.1	1.6

The results of susceptibility and resistance of transformed *E. coli* against rifampicin were shown in terms of MIC in Table VII. The data clearly showed that four strains (Nos. 6, 22 ,23 and 25) kept chromosome-mediated resistance.

4. Resistance test to anti-microbial agents

The MIC values of nine anti-tuberculosis agents against the four chromosome-mediated resistant strains and the parent strain were shown in Table VIII.

The four mutants showed that their resistance to capreomycin was slightly increased, and that their resistance to rifampicin was greatly increased, but their resistance to the other antibiotics were the same as the parent. The results indicate that these strains may be effective when they are orally given simultaneously with the drugs.

The MIC values of six derivatives of penicillins to the four strains were shown in Table IX. Although their resistance to ampicillin and metampicillin was somewhat increased, it was not significant for practical use. The strains may be useful only for carbenicillin when the latter is administered by injection.

Table X. MIC (ug/ml) of cephalosporins against four chromosome mediated resistant strains of *Clostridium butyricum*

Cephalosporins \ Strain No.	6	22	23	25	Parent
Cephalexin	6.3	6.3	6.3	6.3	3.1
Cephazolin	0.4	0.4	0.4	0.4	0.4
Cephaloridine	6.3	6.3	6.3	6.3	6.3
Cephatrizine	0.8	0.4	0.8	0.4	1.6
Ceptizoxime	25	50	50	50	1.6
Cefamandole	1.6	1.6	1.6	1.6	1.6
Cephadroxil	3.1	6.3	6.3	6.3	3.1
Cefuroxime	12.5	12.5	12.5	12.5	3.1
Moxalactam	12.5	25	25	25	3.1

Table XI. MIC (ug/ml) of general antibiotics against four chromosome-mediated resistant strains of *Clostridium butyricum*

Cephalosporins \ Strain No.	6	22	23	25	Parent
Minocycline	0.05	0.05	0.05	0.05	0.05
Chlorampenicol	1.6	1.6	1.6	1.6	0.1
Erythromycin	0.4	0.4	0.4	0.4	0.4
Gentamicin	400	400	400	400	100

The MIC values of nine cephalosporin derivatives to the four strains were shown in Table X. The data showed that their resistance to moxalactam was slightly increased, that the resistance to ceptizoxime was significantly increased, and that the resistance to other six derivatives was negligible.

The MIC values of four frequently used antibiotics to the four strains were shown in Table XI. The results showed that their resistance to chloramphenicol was slightly enhanced.

CONCLUSION

Since *Clostridium butyricum* Miyairi II 588 was very sensitive to rifampicin, its resistance to the drug was induced by using NTG mutagenesis. Among the resistant mutants, nine strains were

selected as potential resistant strains. Four of them were proven to have resistnace mediated by their chromosome but not by the inherent plasmid. They were found to be especially resistant to rifampicin and ceptizoxime. The four mutants would be effective even when they are orally administered simultaneously with the nine anti-tuberculosis agents.

ACKNOWLEDGMENT

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