Arch. Pharm. Res. 12(3), 176-180 (1989)

The Cloning of MLS Antibiotics Inducible Resistance Gene

Eung-Chil Choi§, Kyung-Won Woo, Jong-Suk Woo, Jin-Hwan Kwak and Byong Kak Kim

College of Pharmacy, Seoul National University, Seoul 151-742, Korea

(Received May 10, 1989)

Abstract☐Four bacterial strains having inducible resistance to erythromycin were isolated from soil samples in Korea and characterized. MLS inducibility was checked in each strain. Cloning of inducible resistance gene(s) has been tried. Four isolates were identified as *B. anthracis, Micrococcus luteus, Streptococcus faecium* and *B. licheniformis,* in which erythromycin, oleandomycin, cirramycin and carbomycin acted as resistance inducers respectively. The resistance gene cloned from *B. licheniformis* 597 strain using pBS 42 vector was found to have a 3.2 kb insert.

Keywords☐MLS antibiotics, inducible resistance, gene cloning, *B. licheniformis.*

Some antibiotics at not only as growth (metabolism) inhibitors in microorganisms but also as controlling substances of gene expression. The control mechamism of gene expression by antibiotics in the inducible resistant bacteria to MLS (macrolide-lincosamide-streptogramin B) antibiotics is one example of them. The molecular studies on controlling mechanism of gene expression are expected to contribute to reduce the appearance of resistant strains to these antibitics. The control mechanism of gene expression in resistance to MLS antibiotics in the inducible strains has been explained by post-transcriptional attenuation (translational attenuation) mechanism. This induction mechanism has been elucidated in ermC gene cloned from the *Staphylococus aureus* plasmid pE194[1-3]. Although several MLS resistance elements have been studied and their DNA sequences have been determined[4-9], it is necessary to clone more MLS resistance genes from the different species of bacteria and to compare them to clarify the control mechanism of gene expression. We have tried to clone new resistance gene(s) grom the four selected strains which were isolated from soil samples in Korea, and a MLS resistance gene was cloned from a *Bacillus licheniformis* 597 strain having the different resistant phenotype compared to the reported strains.

MATERIALS AND METHODS

Bacteria and plasmid

Four strains of bacteria (590, 597, CT-4, and TR-1) showing inducible resistance to erythromycin, which were isolated from soil samples in Korea, *E. coli* CSH 26[11] and *B. subtilis* UOTO 277[12] were used. For cloning purpose a shuttle vector pBS42 (between *E. coli* and *B. subtilis*) was also used.

Resistance induction

Induced resistance to MLS antibiotics by MLS was ckecked by agar media method and liquid culture method as described previously[13]. 1) Agar media containing high level of MLS which usually did not act as inducer were prepared, and test microorganism was inoculated on the agar. MLS antibiotics disks were loaded. The microorganism around disks could grow if MLS resistance was induced by the drug contained in disks. D-form inhibition zone by antiboitic disk on agar media having no antibiotics was another indication of resistance induction. 2) The bacteria was cultured on AC broth containing sub-inhibitory concentration of MLS, and the culture was used to inoculate fresh AC broth which contained high level of MLS antibiotics. The good growth was indication of induction.

Identification of the isolated strains

The physiological, morphological, cultural cha-

§ To whom all correspondence should be addressed.

Table I. The physiological, morphological and cultural characteristics of the isolated strains.

	590	CT-4	TR-1	597	*B. subtilis* ATCC 6633
Production of catalase	+	+	+	+	+
VP test	+	−	−	+	+
MR test	−	−	−	−	−
Growth in anaerobic agar	+	−	+	+	−
Growth in 7% NaCl	+	+	+	+	+
Acid and gas from	acid +	−	NT	+	+
Glucose	gas −	−	NT	−	−
Reduction of NO_3^- to NO_2^-	+	−	−	+	+
Starch hydrolysis	+	+	−	+	+
Citrate utilization	+	+	−	+	+
Indole production	−	−	−	−	−
Gelatin hydrolysis	+	+	+	+	+
Urease activity	+	+	NT	+	+
Growth in PLET medium	+	NT	NT	−	−
Gram staining	+	+	+	+	+
Spore formation	+	−	−	+	+
Acid from sorbitol	NT	NT	−	−	NT
Acid from Gycerol	NT	NT−	−	−	NT

NT: not tested

racteristics of each strain were determined. The test results were listed on Table I. The genera and species of each strain were determined according to the guide of 1) Manual for the identification of medical bacteriology[14). 2) Bergey's manual of systematic bacteriology[15), and Bergey's manual of determinative bacteriology[16).

Cloning of resistant genes

Total DNAs were prepared from each strain and digested, partially or completely by EcoRI or BamHI. The digested DNAs were ligated to pBS42. *E. coli* CSH26 was transformed with the ligated plasmids[17). *E. coli* libraries were prepared. *B. subtilis* UOTO 277 was transformed with the plasmids prepared from the *E. coli* libraries[18). *B. subtilis* having MLS resistance gene was selected on both erythromycin and chloramphenicol agar medium.

Table II. Resistance induction to MLS in the isolated strains.

Strains	Inducers	Induced Resistance
Bacillus anthracis 590	EM. OM. CIR.	EM. OM. CIR. KIT. CAR. MAR.
Streptococcus faecium TR-1	EM. OM.	EM. OM. CAR. KIT. CLN. MAR.
Bacillus licheniformis 597	EM. OM. CIR.	EM. OM. CIR. TYL. CHAL.
Micrococcus luteus CT-4	EM. OM. CAR. CIR.	EM. OM. CIR. TYL. CHAL.

EM: erythromycin, OM: oleandomycin, CIR: cirramycin, CAR: carbomycin, KIT: kitasamycin, MAR; maridomycin, CLN: clindamycin, TYL: tylosin, CHAL: chalcomycin

RESULTS AND DISCUSSION

Identification of the isolated strains

The physiological, morphological, cultural characteristics of four strains were determined and results are summarized at Table I. Strain 590 was a Gram-positive rod and formed spore. This bacterium could grow aerobically as well as anaerobically, and form catalase. Especially this strain could grow in PLET medium. So strain 590 was identified as *Bacillus anthracis.* Strain CT-4 was a Gram-positive coccus but did not grow in anaerobic condition. This strain could form neither acid nor gas from glucose. It could not reduce nitrate to nitrite. On the basis of these test results, strain CT-4 was classified as *Micrococcus luteus.* Strain TR-1 was determined as a Gram-positive coccus which could grow aerobically as well as anaerobically. All MR, VP and nitrate reduction test were negative. The strain couldn't use citrate as a carbon source, and didn't form acid from both sorbitol and glycerol. Therefore this strain was identified as *Streptococcus faecium.* Strain 597 was a Gram-positive sporulating bacillus and could grow aerobically and anaerobically. It was a catalase positive strain. The strain could be classified as *B. licheniformis.*

Resistance induction

The isolates were tested for MLS resistance in-

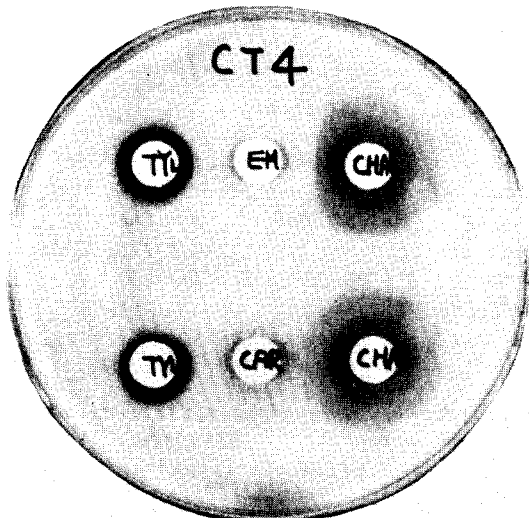

Fig. 1. D-shape inhibition zone in CT-4 strain.

ducibility by agar media method and liquid culture method. Table II shows the results. In strains 590 and 597, resistance to MLS antibiotics could be induced by either erythromycin or the closely related macrolide oleandomycin. Cirramycin could also induce resistance to MLS in these strains. In strain TR-1, resistance to MLS could be induced by either erythromycin or oleandomycin. In CT-4 strain not only erythromycin, oleandomycin, cirramycin but also carbomycin could induce resistance. Carbomycin inducibility in naturally occurrring isolates has not been reported.

Fig. 1 shows the resistance inducibility of both erythromycin and carbomycin in strain CT-4 agar disk method. The D-shape inhibition zone by the chalcomycin disk means that the resistance to chalcomycin was induced by erythromycin. Although the D-shape inhibition zone by the chalcomycin disk neighboring the carbomycin disk wasn't clear, the possibility of resistance induction by carbomycin was high. Especially the resistance induction to carbomycin by carbomycin was strongly expected because the inhibition zone by carbomycin disk wasn't almost identifiable.

Fig. 2 shows the inducibility of erythromycin resistance in strains 590 and TR-1 by liquid culture method. The culture grown in low concentration of erythromycin could grow in the liquid medium containing high concentration of either erythromycin or kitasamycin or carbomycin.

Cloning of resistance gene

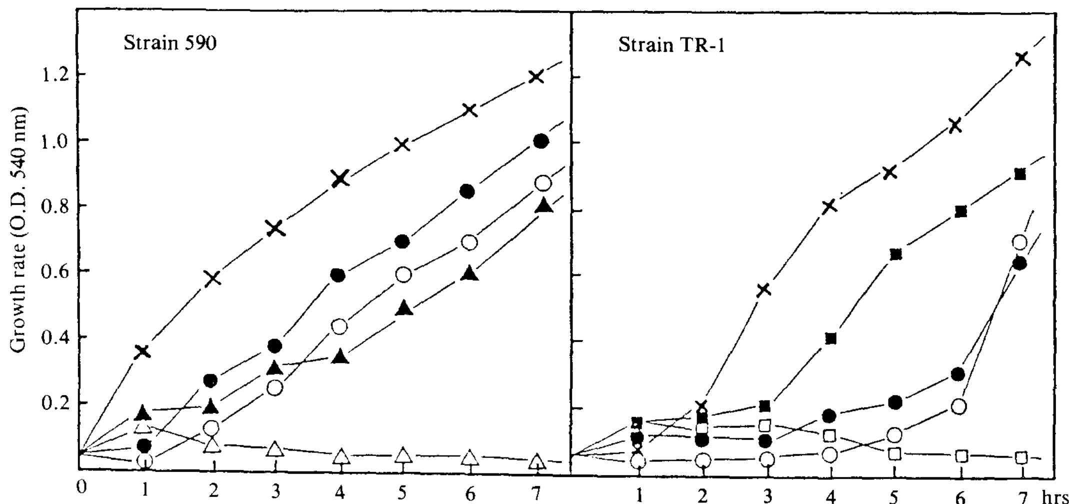

Fig. 2. Resistance inducibility of macrolide antibiotics to MLS in strains 590 and TR-1.

× : Control culture (no antibiotics)
● : Precultured with 1 μg/ml EM, cultured with 100μg/ml EM
○ : Not precultured, cultured with 100μg/ml EM
▲ : Precultured with 1 μg/ml EM, cultured with 100μg/ml KIT
△ : Not precultured, cultured with 100μg/ml KIT
■ : Precultured with 1 μg/ml EM, cultured with 100μg/ml CAR
□ : Not precultured, cultured with 100 μg/ml CAR

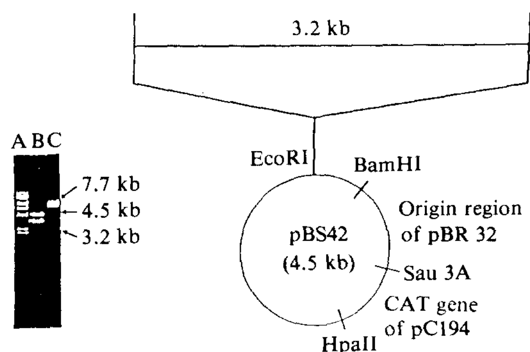

Fig. 3. Resistance gene cloning into pBS42 from *B. licheniformis* strain 597.

The recombinant plasmid was designated as pEC1001.
A: Lambda DNA digested with HindIII
B: pEC1001 DNA digested with EcoRI
C: pEC1001 DNA digested with BamHI

Total chromosomal DNA isolated from strain 597 was completely digested with EcoRI and ligated to pBS 42 plasmid cut with EcoRI. The ligation mixture was used to transform *E. coli* CSH26. Transformants were selected by chloramphenicol and about 5,000 transformants were collected. Plasmid DNA prepared from *E. coli* library was used to transform *B. subtilis* UOTO277. Three transformants showing both erythromycin and chloramphenicol resistance were selected and resistance inducibility to MLS antibiotics was determined. The resistance inducibility patterns determined by agar disk method in these transformants were identical with that of strain 597. The recombinant plasmid was designated as pEC 1001. Fig. 3 shows digestion pattern of pEC1001 with two restriction endonucleases; lane C shows a 7.7 kb linear fragment after BamHI digestion whereas lane B shows two fragments, 3.2 kb and 4.5 kb band after EcoRI digestion. Therefore the pEC1001 contains about the 3.2 kb insert. The 3.2 kb insert size would be enough for one complete gene. Considering the resistance inducibility and the insert size of pEC1001, MLS resistance gene seems to be located on the 3.2 kb DNA fragment. ermD, a MLS resistance element was cloned from *Bacillus licheniformis* strain 749[19] and its DNA sequence was determined[4]. The phenotype of inducible resistance in *B. licheniformis* strain 597 was compared with that in *B. licheniformis* strain 749 and it was found those phenotypes were different. Although the possibility that the homology between the cloned gene from strain 597 and ermD is high, it is thought the newly cloned gene has the partially different DNA base sequences, compared with that of ermD.

ACKNOWLEDGEMEN

This research was supported by GE program, the Ministry of Education, Korea, 1987 via the Institute for Molecular Biology and Genetics, Seoul National University. We acknowledge with gratitude the support.

LITERATURE CITED

1 Gryczan, T., Grandi, G., Hahn, J., Dubnau D.: Conformational alteration of mRNA structure and the posttranscriptional regulation of erythromycin-induced drug resistance. *Nucleic Acids Res.* **8,** 6081 (180).
2. Horinouchi, S. and Weisblum, B.: Post-transcriptional modification of messenge RNA conformation: Mechanism of erythromycin inducible resistance. *Proc. Natl. Acad. Sci. U.S.A.,* **77,** 7079 (1981).
3. Mayford, M. and Weisblum, B.: Messenger RNA from *Staphylococcus aureus* that specifics Macrolide-Lincosamide-Streptogramin resistance: Demonstration of its conformation and of the leader peptide it encodes. *J. Mol. Biol.* **185,** 769 (1985).
4. Gryczan, T., Israeli-Reches, T., Del Bue, M. and Dubnau, D.: DNA sequence and regulation of ermD, a macrolide-lincosamide-streptogramin B resistance element from *Bacillus licheniformis. Mol. Gen. Genet.* **194,** 349 (1984).
5. Murphy, E.: Nucleotide sequence of ermA, a macrolide-lincosamide-streptogramin B determinant in *Staphylococcus aureus. J. Bacteriol.* **162,** 633 (1985).
7. Roberts, A.N., Hudson, G.S. and Brenner, S.: An erythromycin-resistance gene from an erythromycin-producing strain of *Arthrobacter* sp. *Gene* **35,** 259 (1985).
8. Uchiyama, H., and Weisblum, B.: N-methyl transferase of *Streptomyces erythraeus* that confers resistance to the macrolide-lincosamide-streptogramin B antibiotics: Amino acid sequence and its homology to cognate R-factor enzymes from pathogenic bacilli and cocci. *Gene* **38,** 103 (1985).

9. Monod, M., Denoya, C. and Dubnau, D.: Sequence and properties of pIM 13. A macrolide-lincosamide-streptogramin B resistance plasmid from *Bacillus subtilis. J. Bacteriol.* **167,** 138 (1986)
10. Horinouchi, S., Byeon, W., and Weisblum, B.: A complex attenuator regulates inducible resistance to macrolide, lincosamide, and streptogramin B antibiotics in *Streptococcus sanguis. J. Bacteriol.* **154,** 1252 (1983).
11. Miller, J.H.: *Experiments in molecular genetics,* Cold Spring Harber Laboratory Press, Cold Spring Harber U.S.A. (1972).
12. Band, L. and Henner, D.J.: *Bacillus subtilis* requires a stringent Shine-Dalgarno region for gene expression. DNA **3,** 17 (1984).
13. Choi, E.C., Woo, J.W., and Weisblum, B.: Inducible resistance to lincosamide antibiotics by lincosamide antibiotics in *Bacillus licheniformis. Yakhak Hoeji* **30,** 317 (1986)
14. Cowan, S.T.: *Manual for the Identification of Medical Bacteria* (2nd ed.), Cambridge University Press (1974).
15. Smeath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G.: *Bergey's Manual of Systematic Bacteriology* Vol. 2, Williams and Wilkins Co. Baltimore pp. 1599 (1986).
16. Buchanan, R.E. and Gibbons, N.E.: *Bergey's Manual of Determinative Bacterilogy,* 8th ed Williams and Wilkins Company Baltimore, pp. 529 (1974).
17. Mandel, M. and Higa, A.: Calcium dependent bacteriophage DNA infection. *J. Mol. Biol.* **53,** 154 (1970).
18. Dubnau, D.: *Experimental Manipulation of Gene Expression.* Academic Press, New York p. 33-51 (1983).
19. Docherty, A., Grandi, G., Grandi, R., Gryczan, T.J., Shivakumar, A.G. and Dubnau, D.: Naturally occurring macrolide-lincosamide-streptogramin B resistance in *Bacillus licheniformis. J. Bacteriol.* **145,** 129 (1981).