BINDING OF 3-*O*-ACETYL-4''-*O*-ISOVALERYLTYLOSIN TO RIBOSOMES FROM A MACROLIDE-RESISTANT STRAIN OF *STAPHYLOCOCCUS AUREUS*

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The resistance of *Staphylococcus aureus* MS-9610 to tylosin and 3-*O*-acetyltylosin was due to the decreased affinity of its ribosome system to these macrolides. However, 3-*O*-acetyl-4''-*O*-isovaleryltylosin was found to bind to ribosomes of the strain about three times more than 3-*O*-acetyltylosin. This binding was not interfered by tylosin and 3-*O*-acetyltylosin. The 4''-*O*-acyl group and the mycinose moiety were suggested to have an important role in the binding of tylosin derivatives to ribosomes of resistant strains.

3-*O*-Acetyl-4''-*O*-isovaleryltylosin has an improved antibacterial activity against macrolide-resistant strains of *Staphylococcus aureus* clinically isolated. As reported previously[1], the macrolide resistance of multiple-drug resistant strains of *S. aureus* can be divided into two types, the decreased sensitivity of ribosomes (Type I) and the decreased uptake (Type II). Among derivatives of macrolide antibiotics tested, 3-*O*-acetyl-4''-*O*-isovaleryltylosin inhibited the growth of both types of resistant strains. In this paper, we will report on the binding of the compound to 70S-ribosomes of Type I strain and the structural requirements for this binding.

Materials and Methods

Strains and Ribosomes

Staphylococcus aureus MS-9610 was isolated from a clinical specimen and its macrolide resistance was not inducible. The degree of the resistance was over 800 μg per ml of erythromycin, josamycin, spiramycin, angolamycin, midecamycin, tylosin and 3-*O*-acetyltylosin and was 100 μg per ml to penicillin G. *Staphylococcus aureus* FDA 209P was used for comparison as a macrolide-sensitive strain. The culture condition was the same as described previously[1].

70S-Ribosomes were prepared from *S. aureus* MS-9610 and FDA 209P by repeated suspension and centrifugation of ribosomes in a solution containing 1 M NH_4Cl, 10 mM tris-buffer, pH 7.8, 10 mM $Mg(OAc)_2$, 60 mM KCl and 6 mM 2-mercaptoethanol as described in a previous paper[1].

Chemicals

[1-^{14}C]Acetyl group was introduced into the C-3 hydroxyl group of tylosin and 4''-*O*-isovaleryltylosin by an enzymatic method using [1-^{14}C]acetyl-coenzyme A as described previously[2]. These labeled derivatives are described as 3-*O*-[1-^{14}C]acetyltylosin and 3-*O*-[1-^{14}C]acetyl-4''-*O*-isovaleryltylosin. Their specific activities were 7.63 and 8.34 (Lot 1) or 10.30 mCi/mmole (Lot 2), respectively.

L-[U-^{14}C]Phenylalanine (424 mCi/mmole) and L-[U-^{14}C]leucine (351 mCi/mmole) were purchased from Radiochemical Centre, Amersham, England; demycarosyl tylosin (desmycosin)[3] and mycamino-

syl tylonolide[4] were prepared according to the method reported previously; 3-*O*-acetyl-4''-*O*-isovaleryldemycinosyl tylosin was prepared from mycaminosyl tylonolide by microbial transformation or from demycinosyl tylosin[5] by chemical modification (the details for the procedure will be reported elsewhere). All other chemicals were of commercial sources and of the highest grade available.

Determination of [^{14}C]Leucine Incorporation into Cellular Macromolecules

Inhibition of [^{14}C]leucine incorporation into cellular macromolecules was determined according to the method of BYFILED *et al.*[6]. The culture of *S. aureus* MS-9610 at an exponential growth phase (90 μl; $OD_{660}=0.2$) containing 0.1 mM of deoxyadenosine was mixed with 10 μl of 5% aqueous methanol containing a test sample. The final concentration of the sample was 25 μM (Fig. 1) and 12.5 μM (Table 2). It was incubated at 37°C for 10 minutes and 10 μl of L-[U-^{14}C]leucine (5 μCi/ml) was added to the cell suspension. After incubation at 37°C for 30 minutes, 100 μl was taken and placed onto a Whatman 3 MM filter paper disc (2.4 cm diameter). The wet discs were immersed in ice-cold 10% trichloroacetic acid (TCA) (5 ml/disc), and processed as reported previously[1]. Radioactivity in cold TCA insoluble materials was determined by a toluene scintillation method.

In Vitro Protein Synthesis on 70S-Ribosomes

In vitro protein synthesis in a system containing washed 70S-ribosomes of *S. aureus* and S-100 from *E. coli* Q-13 (RNase I$^-$) was conducted as reported by MAO[7] with minor modifications and the condition as reported in a previous paper[1].

Determination of Tylosin Derivative-Ribosome Complexes

The formation of ^{14}C-labeled tylosin derivative-ribosome complexes in staphylococcal strains was determined by a gel-filtration method[8] and membrane filtration method as reported by PESTKA[9] and MAO & PUTTERMAN[10].

1) Sephadex G-200 Gel Column Chromatography: Ribosomes (20 μl, about 5.2 OD_{260} units) were mixed with 10 μl of 3-*O*-[1-^{14}C]acetyltylosin (0.61 μg, 6.4×10^{-10} mole, 7.63 mCi/mmole) or 3-*O*-[1-^{14}C]-acetyl-4''-*O*-isovaleryltylosin (0.46 μg, 4.4×10^{-10} mole, 10.30 mCi/mmole) solution in a buffer (total volume 100 μl; 50 mM tris-buffer, pH 7.8, 50 mM NH_4Cl, 16 mM $Mg(OAc)_2$ and 100 μM dithiothreitol which was described below as the standard buffer and the mixtures were incubated at 37°C for 20 minutes. ^{14}C-Labeled tylosin derivative-ribosome complexes were subjected to Sephadex G-200 gel column chromatography (column size; 0.6×20 cm, 0.25 ml/fraction) preequilibrated with the standard buffer, using the same buffer for elution.

2) Millipore Membrane Filtration: Ribosomes (20 μl, 5.2 OD_{260} units for MS-9610 and 7.6 OD_{260} units for FDA 209P) were mixed with 5 μl of 3-*O*-[1-^{14}C]acetyltylosin (7.63 mCi/mmole, 3.05 μg) or 10 μl of 3-*O*-[1-^{14}C]acetyl-4''-*O*-isovaleryltylosin (8.34 mCi/mmole, 2.50 μg) solution in the standard buffer (total volume 100 μl) and the mixture were incubated at 37°C for 20 minutes. The mixture was diluted with 3 ml of a cold standard buffer containing the corresponding non-radioactive tylosin derivative at 50 μl/ml, filtered by gentle suction through a Millipore membrane filter (HA, 0.45 μm pore size, 2.4 cm diameter). The membrane filter was presoaked with the cold standard buffer described above. This pretreatment of the filter with a non-labeled derivative decreased the background value. The value without ribosomes was taken as the background value. The ^{14}C-labeled tylosin derivative-ribosome complexes on the membrane filter were washed twice with 3 ml of the above cold standard buffer and the radioactivity remaining on the filter disc was measured by a liquid-scintillation method.

Interference of the Binding of Labeled Tylosin Derivatives to Ribosomes by Unlabeled Antibiotics

The inhibition of the binding of labeled tylosin derivatives to MS-9610 ribosomes by unlabeled antibiotics structurally related and unrelated was examined by the abilities of the latter to replace the former in the formation of the ribosome complexes. Ribosomes (40 μl, 6.9 OD_{260} units) were mixed with an indicated amount of an unlabeled compound (20 μl) in the standard buffer (120 μl, Fig. 3, Table 2). After preincubation at 37°C for 10 minutes, 20 μl of 3-*O*-[1-^{14}C]acetyltylosin (1.22 μg, 1.27×10^{-9} mole, 7.63 mCi/mmole) or 3-*O*-[1-^{14}C]acetyl-4''-*O*-isovaleryltylosin (0.92 μg, 8.8×10^{-10} mole, 10.30 mCi/mmole) was added to the reaction mixture. After incubation at 37°C for 20 minutes, the reaction mixture was diluted, filtered and washed as described in a previous paragraph.

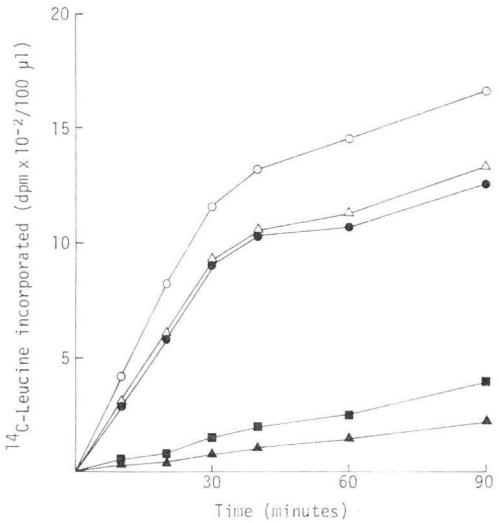
Results and Discussion

Inhibition of Protein Synthesis by Tylosin Derivatives

The effect of tylosin derivatives on *in vivo* protein synthesis in Type I macrolide-resistant *Staphylococcus* strain, MS-9610, is shown in Fig. 1. 3-*O*-Acetyl-4''-*O*-isovaleryltylosin and 4''-*O*-isovaleryltylosin showed a strong inhibition. This was in a good agreement with the data on growth inhibitory activities of tylosin derivatives. The dose-dependent effect of tylosin derivatives on protein synthesis on ribosomes[1] prepared from macrolide-resistant (MS-9610) or -sensitive (FDA 209P) strains was examined. Fifty percent inhibition concentrations (ID_{50}) of tylosin, 3-*O*-acetyltylosin, 4''-*O*-isovaleryltylosin and 3-*O*-acetyl-4''-*O*-isovaleryltylosin for the protein synthesis on MS-9610 ribosomes were 9.20, >20, 0.90 and 0.80 μg/ml, respectively, and for the protein synthesis on ribosomes of FDA 209P were 0.19, 0.39, 0.78 and 0.65 μg/ml, respectively. Thus, MS-9610 ribosomes were found to be resistant to tylosin and 3-*O*-acetyltylosin but sensitive to 3-*O*-acetyl-4''-*O*-isovaleryltylosin or 4''-*O*-isovaleryltylosin.

Fig. 1. Inhibition by tylosin derivatives (25 μM) on [^{14}C]leucine incorporation into acid-insoluble fractions in intact cell system of *Staphylococcus aureus* MS-9610 (macrolide-resistant).

Control (none, ○), tylosin (●), 3-*O*-acetyltylosin (△), 4''-*O*-isovaleryltylosin (▲), 3-*O*-acetyl-4''-*O*-isovaleryltylosin (■).



Formation of Tylosin Derivative-Ribosome Complexes

The amounts of binding of tylosin derivatives to ribosomes of MS-9610 and FDA 209P determined by a membrane filter method are shown in Table 1. Thirteen pmole of 3-*O*-[1-^{14}C]acetyltylosin bound to 1 OD_{260} unit of MS-9610 ribosomes and this amount was one third of the amount of 3-*O*-[1-^{14}C]acetyltylosin bound to FDA 209P ribosomes. The amount of 3-*O*-[1-^{14}C]acetyl-4''-*O*-isovaleryltylosin bound to MS-9610 ribosomes was three times more than that of 3-*O*-[1-^{14}C]acetyltylosin. This suggests that a stronger inhibition of protein synthesis by 3-*O*-acetyl-4''-*O*-isovaleryltylosin is due to the greater ability

Table 1. The formation of tylosin derivative-ribosome complexes in a macrolide-sensitive strain (FDA 209P) and -resistant strain (MS-9610) of *Staphylococcus aureus*.

Source of ribosome*1	Tylosin derivative	The amount bound to ribosomes*2 (pmole/OD_{260})	MIC (μg/ml)	ID_{50}*3 (μg/ml)
FDA 209P	3-*O*-Acetyltylosin	38	0.78	0.4
	3-*O*-Acetyl-4''-*O*-isovaleryltylosin	185	1.56	0.7
MS-9610	3-*O*-Acetyltylosin	13	>1,600	>20
	3-*O*-Acetyl-4''-*O*-isovaleryltylosin	39	100	0.80

*1 FDA 209P (7.6 OD_{260} units), MS-9610 (5.2 OD_{260} units)

*2 The amount of a compound bound to ribosomes was determined using 3-*O*-[1-^{14}C]acetyltylosin (7.63 mCi/mmole, 5 μl, 3.05 μg) and 3-*O*-[1-^{14}C]acetyl-4''-*O*-isovaleryltylosin (8.34 mCi/mmole, 10 μl, 2.50 μg) as described in the Methods.

*3 50% Inhibition concentration against poly (U)-directed poly Phe synthesis in a cell-free system.

Fig. 2 Binding of [^{14}C]tylosin derivatives to 70S-ribosomes of MS-9610 cells.
[^{14}C]Tylosin derivative-ribosome complexes were eluted from Sephadex G-200 column.
(a) 3-*O*-[1-^{14}C]Acetyltylosin.
(b) 3-*O*-[1-^{14}C]Acetyl-4''-*O*-isovaleryltylosin.

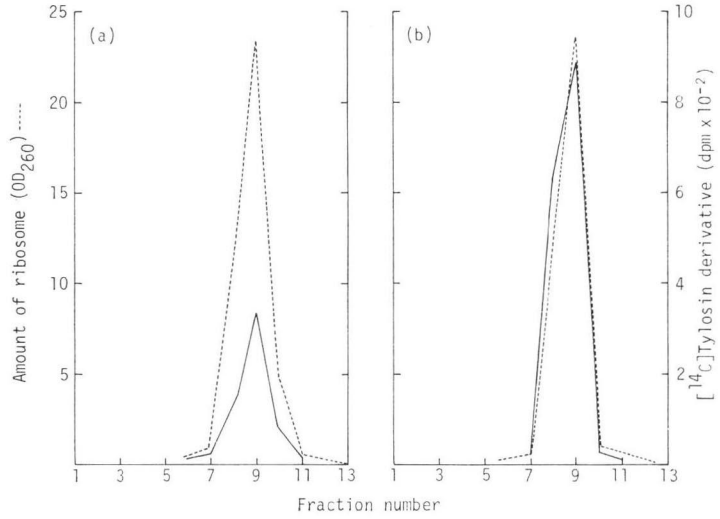
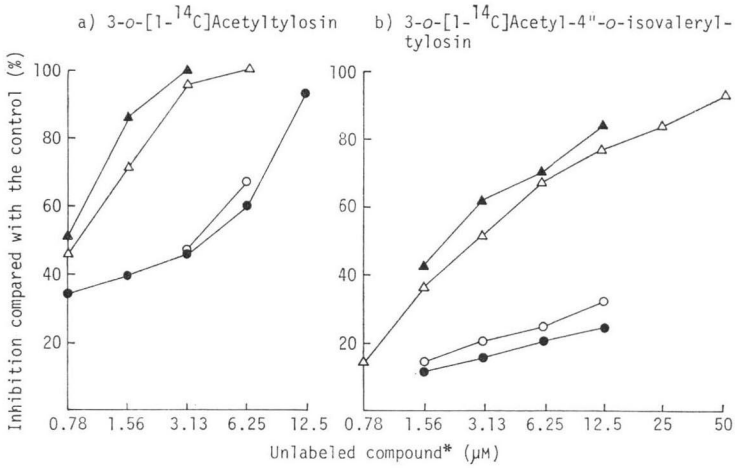


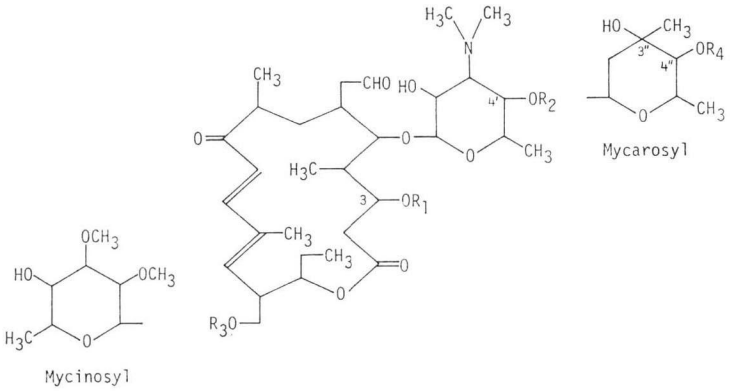
Fig. 3. Effect of unlabeled compounds on the binding of 3-*O*-[1-^{14}C]acetyltylosin (6.4 μM) or 3-*O*-[1-^{14}C]-acetyl-4''-*O*-isovaleryltylosin (4.4 μM) to 70S-ribosomes of MS-9610 cells.
* Unlabeled compound: tylosin (○), 3-*O*-acetyltylosin (●), 4''-*O*-isovaleryltylosin (▲), 3-*O*-acetyl-4''-*O*-isovaleryltylosin (△).



of this derivative to bind to MS-9610 ribosomes. The same result was also observed by the gel filtration method (Fig. 2).

While, in spite of having almost the same antibacterial activities or inhibitory activities in a cell-free protein system, the amount of 3-*O*-[1-^{14}C]acetyl-4''-*O*-isovaleryltylosin bound to FDA 209P ribosomes was 4.9 times more than that of 3-*O*-[1-^{14}C]acetyltylosin (Table 1). This indicates that the increase of binding affinity to ribosomes from a macrolide-sensitive strain does not necessarily correspond to high antibacterial activity.

Fig. 4. Chemical structure of compounds related to 3-*O*-acetyl-4''-*O*-isovaleryltylosin.



Compound	R_1	R_2	R_3	R_4
3-*O*-Acetyl-4''-*O*-isovaleryltylosin	$COCH_3$	mycarosyl	mycinosyl	$COCH_2CH(CH_3)_2$
3-*O*-Acetyltylosin	$COCH_3$	mycarosyl	mycinosyl	H
4''-*O*-Isovaleryltylosin	H	mycarosyl	mycinosyl	$COCH_2CH(CH_3)_2$
Tylosin	H	mycarosyl	mycinosyl	H
3-*O*-Acetyl-4''-*O*-isovaleryl-demycinosyl tylosin	$COCH_3$	mycarosyl	H	$COCH_2CH(CH_3)_2$
Demycarosyl tylosin	H	H	mycinosyl	—
Mycaminosyl tylonolide	H	H	H	—

To compare the affinity of 3-*O*-acetyl-4''-*O*-isovaleryltylosin to MS-9610 ribosomes with that of 3-*O*-acetyltylosin, the inhibition between them in ribosome binding was examined at various concentrations, as shown in Fig. 3. The binding of 3-*O*-[1-^{14}C]acetyltylosin was almost completely inhibited by 3-*O*-acetyl-4''-*O*-isovaleryltylosin at one half concentration of the former, while the binding of 3-*O*-[1-^{14}C]acetyl-4''-*O*-isovaleryltylosin was inhibited only 25% by the addition of two fold concentration of tylosin or 3-*O*-acetyltylosin. This indicates that the affinity of 3-*O*-acetyl-4''-*O*-isovaleryltylosin is much higher than that of tylosin. The binding sites of tylosin derivatives on MS-9610 ribosomes and the nature of this ribosomes will be reported elsewhere. The large difference between the MIC (100 μg/ml) and ID_{50} (0.8 μg/ml) values of 3-*O*-acetyl-4''-*O*-isovaleryltylosin on MS-9610 strain may indicate that there is an internal inhibition of the passage of the derivative caused by its high binding ability to cellular components.

Structural Relationships among Tylosin Derivatives in Binding to Ribosomes

Structural relationships in binding to ribosomes were examined by interference between compounds in binding to ribosomes. Compounds used in this experiment are shown in Fig. 4. The results are listed in Table 2 together with the results of inhibitory activities of the compounds against protein synthesis in intact cells and a cell-free system of MS-9610 cells. In case of the compounds lacking an acyl group at the C-4'' position of tylosin analogues, such as 3-*O*-acetyltylosin, tylosin, demycarosyl tylosin and mycaminosyl tylonolide, the ability to bind to ribosomes was markedly lower compared with tylosin derivatives having an acyl group at 4''-position. In spite of having an acyl group at the 4''-position, 3-*O*-acetyl-4''-*O*-isovaleryl-demycinosyl tylosin also had low binding ability, indicating that the mycinose moiety is necessary for binding to ribosomes of MS-9610 strain. The requirement of mycinose was also suggested

Table 2. The influence of various antibiotics on the binding of 3-*O*-acetyl-4''-*O*-isovaleryltylosin to ribosomes of MS-9610 cells in comparison with their effects on protein synthesis *in vivo* and *in vitro* and growth inhibition.

Compound	% Inhibition by a compound (12.5 μM) of		ID_{50}*2 (μg/ml)	MIC (μg/ml)
	Binding of [^{14}C]-3Ac4''-iVTS (4.4 μM) to 70S-ribosomes*1	Protein synthesis by intact cells		
3-*O*-Acetyl-4''-*O*-isovaleryltylosin	79	55	0.8	100
4-*O*-Isovaleryltylosin	84	61	0.9	100
3-*O*-Acetyl-4''-*O*-isovaleryl-demycinosyl-tylosin	19	14	>20	>1600
3-*O*-Acetyltylosin	24	10	>20	>1600
Tylosin	30	11	9.3	1600
Demycarosyl tylosin	24	−35	>20	>1600
Mycaminosyl tylonolide	13	9	>20	>1600
Josamycin	8	10	>20	>1600
Spiramycin	0	7	>20	>1600
Angolamycin	40	15	12.3	1600
Kanamycin	−6	—	—	1.56
Tetracycline	−10	—	—	6.25
Streptomycin	−3	—	—	1.56
Chloramphenicol	5	—	—	6.25

*1 70S-ribosomes (6.9 OD_{260} units)
[^{14}C]-3Ac4''iVTS: 3-*O*-[1-^{14}C]acetyl-4''-*O*-isovaleryltylosin.

*2 50% Inhibition concentration in *in vitro* protein synthesis with 70S ribosomes from *S. aureus* MS-9610.

by a high binding ability of angolamycin compared with josamycin and spiramycin. Angolamycin has mycinose at the same position of the aglycone as tylosin, while josamycin and spiramycin have not. Compared with 3-*O*-acetyl-4''-*O*-isovaleryltylosin, the binding ability of angolamycin was significantly low. Introduction of acyl group into the C-3 hydroxyl group of the aglycone did not enhance the binding ability to ribosomes.

Other antibiotics known as bacterial protein synthesis inhibitors such as, chloramphenicol, streptomycin, tetracycline and kanamycin, did not inhibit with the binding of 3-*O*-[1-^{14}C]acetyl-4''-*O*-isovaleryltylosin to ribosomes.

References

1) Tsuchiya, M.; K. Suzukake, M. Hori, T. Sawa, R. Okamoto, H. Nomura, H. Tsunekawa, T. Inui, T. Takeuchi & H. Umezawa: Studies on the effects of 3-*O*-acetyl-4''-*O*-isovaleryltylosin against multiple-drug resistant strains of *Staphylococcus aureus*. J. Antibiotics 34: 305~312, 1981
2) Okamoto, R.; T. Fukumoto, H. Nomura, K. Kiyoshima, K. Nakamura, A. Takamatsu, H. Naganawa, T. Takeuchi & H. Umezawa: Physico-chemical properties of new acyl derivatives of tylosin produced by microbial transformation. J. Antibiotics 33: 1300~1308, 1980
3) Hamill, R. L.; M. E. Haney, Jr., M. Stamper & P. F. Wiley: Tylosin, a new antibiotic. II. Isolation, properties, and preparation of desmycosin, a microbiologically active degradation product. Antibiot. Chemother. 11: 328~334, 1961
4) Morin, R. B. & M. Gorman: The partial structure of tylosin, a macrolide antibiotic. Tetrahedron Lett. 1964: 2339~2345, 1964

5) BALTZ, R. H. & E. T. SENO: Properties of *Streptomyces fradiae* mutant blocked in biosynthesis of the macrolide antibiotic tylosin. Antimicr. Agents Chemother. 20: 214~225, 1981
6) BYFILED, J. E. & O. H. SCHERBAUM: A rapid radioassay technique for cellular suspensions. Anal. Biochem. 17: 434~443, 1966
7) MAO, J. C.-H.: Protein synthesis in a cell-free extract from *Staphylococcus aureus*. J. Bacteriol. 94: 80~86, 1967
8) SHIMIZU, M.; T. SAITO, H. HASHIMOTO & S. MITSUHASHI: Spiramycin resistance in *Staphylococcus aureus*. Decrease in the ribosomal affinity of spiramycin in resistant staphylococci. J. Antibiotics 23: 63~67, 1970
9) PESTKA, S.: Binding of [^{14}C]erythromycin to *Escherichia coli* ribosomes. Antimicrob. Agents Chemother. 6: 474~478, 1974
10) MAO, J. C.-H. & M. PUTTERMAN: Accumulation in Gram-positive and Gram-negative bacteria as a mechanism of resistance to erythromycin. J. Bacteriol. 95: 1111~1117, 1968